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UNIVERSITY OF ALBERTA

NEURAL CELL ADHESION MOLECULE (N-CAM) IN DEVELOPING FEATHERS: ANALYSIS OF STRUCTURAL VARIANTS AND PERTURBATION OF FUNCTION

by

Randall Glenn Marsh

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

Edmonton, Alberta
Spring 1992

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Date: April 23, 1992

We must not conceal from ourselves the fact that the causal investigation of organisms is one of the most difficult, if not the most difficult problem which the human intellect has attempted to solve, and that this investigation, like every causal science, can never reach completeness, since every new cause ascertained only gives rise to fresh questions concerning the cause of this cause.

WILHELM ROUX (1894)

Seek simplicity and distrust it.

ALFRED NORTH WHITEHEAD (1919)

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

Neural Cell Adhesion Molecule (N-CAM) in Developing Feathers: Analysis of Structural Variants and Perturbation of Function

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ABSTRACT

The neural cell adhesion molecule (N-CAM) is a family of proteins that mediate cell-cell contact. The individual N-CAM proteins may have different functions in regulating cell-cell interaction during development, but these differences are not understood. A first step in understanding the functions of the different forms of N-CAM is to correlate the expression of these forms with different morphogenetic events. This thesis describes (a) the structural forms of N-CAM present in developing chicken feathers and, (b) the effects of perturbing N-CAM-mediated adhesion in this system.

N-CAM in skin appears as a 145 kD protein, encoded by a 6.4 kb mRNA, compared to the 140 kD N-CAM protein and 6.1 kb mRNA in brain. PCR analysis shows the 6.4 kb skin mRNA actually consists of two transcripts, with and without a 93-bp insert between exons 12 and 13. Because only a single mRNA band is detected in skin, the 93-bp insert can not be the basis for the 300-bp larger size of the two skin mRNAs. Their larger size may be due to use of an alternative transcriptional start site. The presence of two mRNAs means that two polypeptides are probably expressed in skin. However, because only a single protein is detected, the 93-bp insert can also not account for the 5 kD larger size of the skin proteins. Their larger size is due to N-linked carbohydrate. Localization of N-CAM and polysialic acid (PSA) in skin reveals that N-CAM is present on epidermal cells as early as E6 and that PSA is expressed at specific times on only a subset of the cells that express N-CAM.

Perturbation of N-CAM-mediated adhesion using an antibody that inhibits N-CAM homophilic binding, and removal of PSA using endoneuraminidase-N, did not cause any apparent changes in formation of feather rudiments or filaments.

In summary, variations in N-CAM mRNA and protein, which may be important in modulating N-CAM function in developing feathers, were discovered. Initial analyses of the effects of varying some of these factors did not provide any insight into the function of these variations.

PREFACE

Embryogenesis in vertebrates proceeds through a series of cellular interactions in which different cells become sorted out and assembled into multicellular groupings that give rise to tissues and organs. The capacity of cells to recognize one another, adhere, and assemble into groups during development depends on interactions between cell surface constituents on apposed cells. Cell adhesion molecules (CAMs) are cell surface proteins that mediate direct contact between the plasma membranes of apposed cells.

Cell adhesion mediated by CAMs is involved in the formation of intercellular junctions, which allow for communication between cells and for the development of physiologically distinct tissue compartments. The formation of intercellular junctions is thought to join cells into groups that exhibit characteristics different from those of single cells. These collectives of cells respond to and generate inductive signals that cause changes in gene expression and subsequently lead to differentiation and the establishment of form. Thus, the processes that are involved in cell adhesion are of prime importance in development, and characterization of these processes should provide insight into how development occurs.

The neural cell adhesion molecule (N-CAM) is a family of proteins that mediate direct cell-cell contact between a variety of cells in the developing embryo. The different N-CAM proteins are generated from a single gene by differential mRNA splicing. The individual N-CAM proteins are expressed at specific times and locations in the

embryo, and are often present on cell groups during inductive interactions. This suggests that the individual N-CAM proteins may have different functions and may play specific roles in inductive interactions during development.

The function of the N-CAM proteins is thought to be modulated by attachment of a specialized carbohydrate moiety called polysialic acid (PSA). PSA is large and negatively charged, and decreases N-CAM-mediated adhesion by preventing contact between plasma membranes of apposed cells. The amount of PSA attached to N-CAM is regulated during development, suggesting that PSA may play an important role in controlling N-CAM-mediated adhesion during morphogenesis. At present, the developmental function of PSA, and of the different N-CAM proteins, is poorly understood.

Feather formation in chicken skin offers a model in which to examine the function of N-CAM-mediated adhesion in the formation and patterning of embryonic cell populations, i.e. the collectives that are thought to be the basic units of development. Feather morphogenesis is characterized by the formation of discrete epidermal and dermal cell groups that interact with one another and differentiate into the mature feather. During feather formation, N-CAM is known to be expressed on cells that are involved in epidermal-dermal induction, on cells that are undergoing division, and on cells that may undergo programmed death. This suggests that differential control of N-CAM-mediated adhesion by selective expression of the N-CAM proteins and of PSA may be involved in regulating these cellular events and in directing feather formation.

This thesis describes the structural forms of N-CAM present, and the effects of perturbing N-CAM-mediated adhesion, during feather morphogenesis. As a background to describing this work, the introduction contains a historical summary of the field of cell adhesion and how N-CAM was isolated, a description of the features of N-CAM and how its adhesive properties are regulated, an overview of the functions that N-CAM-mediated adhesion may have in development, and a description of feather formation and N-CAM expression during this process. The following two sections of the thesis describe the forms of N-CAM present in developing feathers and a preliminary investigation into the effects of perturbing N-CAM-mediated adhesion in this system. The final section is a discussion of these results in terms of possible functions of N-CAM-mediated adhesion in developing feathers and future directions for research.

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Materials for preparation of endoneuraminidase-N were kindly supplied by Dr. Eric Vimr, Department of Veterinary Pathology, University of Illinois at Urbana-Champaign.

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ABBREVIATIONS

bp	base pair
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	bovine serum albumin
CAM	cell adhesion molecule
ChAM graft	chorioallantoic membrane graft
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
DAB	3,3' diaminobenzidine tetrahydrochloride
DMSO	dimethyl sulphoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
endo-F	endoglycosidase-F
endo-N	endoneuraminidase-N
FITC	fluorescein isothiocyanate
FLS	filament-like structure
HNK-1	human natural killer cell 1
HRP	horseradish peroxidase
kb	kilobase
kD	kilodalton
L-CAM	liver cell adhesion molecule
mAb	monoclonal antibody
MSD1	muscle specific domain 1
M_r	relative molecular weight
NBT	nitro blue tetrazolium
N-CAM	neural cell adhesion molecule
PBS	phosphate buffered saline

PMSF	phenylmethanesulfonyl fluoride
PNA	peanut lectin
PSA	polysialic acid
RACE	rapid amplification of cDNA ends
RITC	rhodamine isothiocyanate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	tris buffered saline
TTBS	TBS with 0.1% Tween 20
VASE (π)	variable domain alternatively spliced exon
WGA	wheat germ agglutinin

CHAPTER I

History and Background

Historical summary of the field of cell adhesion

The central problem of developmental biology is to understand the mechanisms by which form is generated and maintained. Early descriptive studies of development in different animals show that embryogenesis proceeds through a series of interactions in which different cells sort-out and assemble into multicellular groupings that give rise to tissues and organs. Each organ has a distinct form, a precisely ordered arrangement of cells, and specific relationships to other organs. Clearly, certain mechanisms must be responsible for holding cells together to allow for segregation and organization of cell groups in the developing embryo, and must also be involved in stabilizing multicellular structures once they have formed.

The first experimental approach to understanding how cells recognize and adhere to one another is attributed to Wilson (1910), who dissociated sponges by forcing them through silk mesh. The dissociated cells re-aggregate into clusters that eventually form into new, intact sponges. Wilson observed that reformation of new individuals depends upon movement of cells within the aggregates so that their appropriate position within the mass is regained. He had thus discovered the ability of cells to self-assemble into structures having a higher level of organization. Later experiments conducted by Galtsoff (1925) show that cells from different sponge species

preferentially aggregate with their own type in a mixture of cells from two species, demonstrating that specific recognition occurs at the level of the individual cell.

Further evidence that species-specific re-aggregation is due to adhesion of cells to their own kind came from the use of a rotary shaker method developed by Moscona (1961). This method allows cell re-aggregates to form as a result of adhesive stability only, and rules out the potential for cell migration that may occur in stationary (non-rotated) systems. When mixtures of different sponge species are rotated together using this method, aggregates consisting of only one cell type are still formed. This confirms the idea that each cell preferentially adheres to its own kind and that recognition occurs at the level of the single cell.

The ability of cells to selectively recognize and adhere to one another is not only a property of invertebrate cells; when cells of developing vertebrate embryos are mixed, they too segregate according to cell type and re-establish their former associations. Townes and Holtfreter (1955) found that when dissociated cells from a whole amphibian embryo or from various tissues are incubated in tissue culture dishes, the cells recombine into aggregates containing several cell types. After several days, cells in these aggregates sort-out into defined groups and form individual cell populations composed of only one cell type. By using embryos from species having cells of different sizes and colors, the behavior of the recombined cells can be followed. Cells within the aggregates are observed to sort-out into defined regions in accord with their original position in the embryo, i.e. epidermal cells outside, mesoderm in the

middle, and endodermal cells inside. In addition, the adhesive affinities of each cell population, and hence their position within the aggregate, is found to change at different developmental stages. This implies that specific interactions between cell populations occur during embryogenesis.

In order to explain the sorting-out of cells seen during development, Steinberg (1970) proposed a model for adhesive interaction that is based on free energy of cell-cell binding. This theory, called the differential adhesion hypothesis, suggests that mixed cells interact to form an aggregate by rearranging themselves into a thermodynamically stable pattern with low free energy. The only requirement for the sorting to occur is for cells to differ in their relative strengths of adhesion. By varying either the amount or type of cellular "glue," or both, the stability of contact between cells can be altered and cells can sort-out due to thermodynamic differences in binding. In theory, the use of relatively few adhesion molecules should be sufficient to allow for sorting and patterning of cell populations during embryogenesis, since each population can preferentially adhere to another by changing the quantity or type of "glue" at specific times and places.

While all of these observations and ideas argue that selective adhesion between cells plays a role in the sorting-out of cell populations during development, none identify the molecules underlying this process. It is not until the late 1970s that the molecular basis for cell-cell adhesion would start to be identified.

The strategy used in searching for molecules involved in cell adhesion is based on an immunological approach in which antisera are produced that block adhesion between cells. The basic idea is that when an antiserum containing an antibody to an adhesion molecule is incubated with cells, it binds to the adhesion sites on the cell surface and blocks adhesion between the cells. Brackenbury et al. (1977) used this idea to raise antisera against cells from chicken neural retina and isolate antisera that inhibit cell-cell aggregation. To purify the specific anti-cell adhesion molecule (CAM) antibodies in the antisera, a neutralization assay is used in which fractions of cell surface proteins are tested for their ability to compete with cells for binding to the anti-CAM antibody. Fractions that prevent binding of cells to the antibody must contain the original CAM that the antibody binds to. These fractions are used to re-immunize rabbits and generate new antisera. By iteration of this procedure, pure antigenic fractions are obtained and used to generate specific anti-CAM antibodies. This type of approach led to the isolation of the neural cell adhesion molecule (N-CAM) (Brackenbury et al., 1977; Thiery et al., 1977), liver cell adhesion molecule (L-CAM) (Bertolotti et al., 1980; Gallin et al., 1983), and neural-glial cell adhesion molecule (Ng-CAM) (Grumet and Edelman, 1984; Grumet et al., 1984a). The remainder of this introduction describes the features and developmental functions of the neural cell adhesion molecule, N-CAM.

Structural characteristics of N-CAM and modulation of its adhesive properties

N-CAM is a family of closely related proteins that mediate calcium-independent, homophilic adhesion between a variety of cell types in the developing embryo (Hoffman et al., 1982; Thiery et al., 1982; Crossin et al., 1985; Hall et al., 1990). In embryonic chicken brain there are three major N-CAM polypeptides with molecular weights of 180, 140, and 120 kD (Murray et al., 1986) (see Fig. I-1). All three proteins are identical from their amino terminus, which is outside the cell, to the region where they are associated with the cell membrane (Murray et al., 1986; Cunningham et al., 1987). This extracellular region of the proteins contains five immunoglobulin-like domains in which the binding site for N-CAM is located; interaction between these domains on two apposed N-CAM molecules forms the basis of N-CAM homophilic binding (Cunningham et al., 1987; Hall et al., 1990). The two larger N-CAM polypeptides are integral membrane proteins and have cytoplasmic domains. The larger of these (180 kD) contains an additional 261 amino acids in its cytoplasmic domain (Cunningham et al., 1987). The smallest polypeptide (120 kD) lacks a membrane spanning region entirely, and is attached to the cell membrane via a phosphatidylinositol lipid anchor (Sadoul et al., 1986; Hemperly et al., 1986b; He et al., 1987).

The different N-CAM polypeptides are generated by alternative splicing of a single gene (Murray et al., 1986) (see Fig. I-2). The N-CAM gene in chicken is approximately 110 kilobases (kb) in length and contains 19 exons (Murray et al., 1986; Hemperly et al., 1986a; Cunningham et al., 1987; Colewell et al., 1991). The first fourteen

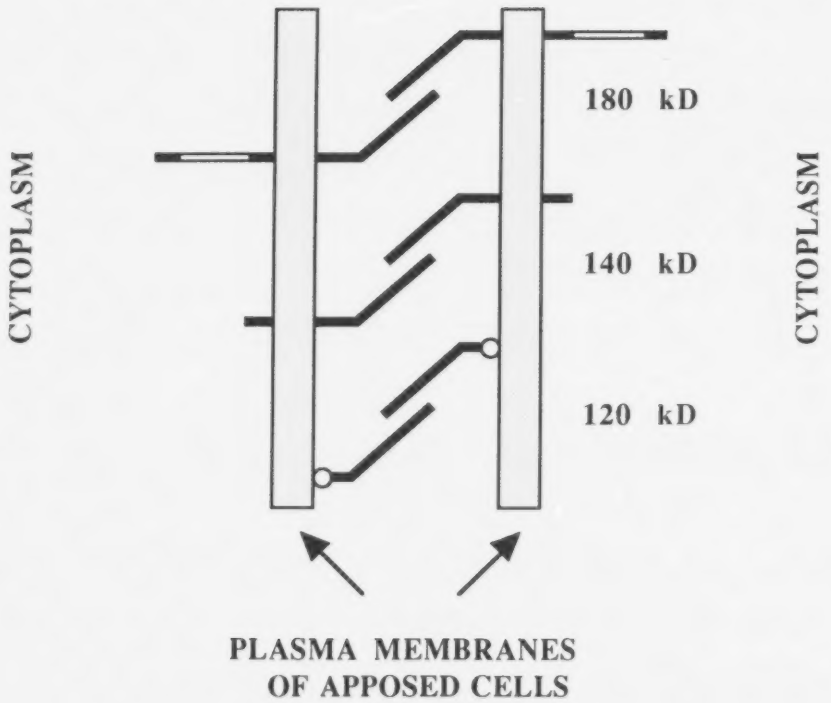


Fig. I-1. Diagramatic representation of the three major N-CAM polypeptides. All three are identical from their amino terminus (outside cell) to the region where they are associated with the plasma membrane. The 180 kD and 140 kD polypeptides are both transmembrane, but the 180 kD polypeptide contains an extra cytoplasmic domain (═══). The 120 kD polypeptide is attached to the plasma membrane by a phosphatidylinositol anchor (O). Interaction between the extracellular domains of the polypeptides on apposed cells forms the basis of N-CAM homophilic binding.

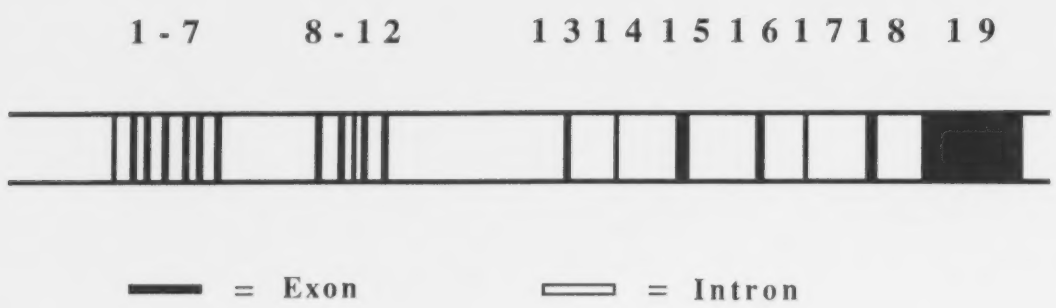
exons of the gene are common to all of the N-CAM proteins; the differences in the individual protein chains arise by alternative splicing of the remaining five exons. Exon 18 is specific to the 180 kD protein and encodes the extra cytoplasmic domain present in this form (Murray et al., 1986). Exon 15 is present only in the 120 kD polypeptide and generates the phosphatidylinositol linkage (Murray et al., 1986; Hemperly et al., 1986b). The size of the mRNAs generated for the brain N-CAM proteins are 6.9, 6.1, and 4.2 kb for the 180, 140, and 120 kD forms, respectively (Murray et al., 1986). These mRNA and protein sizes are specific for brain. Other tissues in the chicken embryo contain different-sized N-CAM mRNAs and polypeptides due to alternative splicing of additional exons and to glycosylation of the proteins, as explained below.

The adhesion mediated by N-CAM is regulated in several ways during development, namely (i) changes in the amount of N-CAM expressed at the cell surface, (ii) changes in the N-CAM proteins being expressed due to differences in mRNA splicing, and (iii) post-translational modification of the N-CAM polypeptides.

(i) Changes in the amount of N-CAM at the cell surface

Variation in the amount of N-CAM present on the cell surface causes large changes in the rate of N-CAM homophilic binding (Hoffman and Edelman, 1983). Membrane vesicles prepared from brain tissue, or vesicles reconstituted from purified N-CAM and lipid, both show large changes in the rate of N-CAM binding (vesicle aggregation) proportional to the surface density of N-CAM. Specifically, aggregation rate of the vesicles increases in proportion

A. Gene Structure



B. Differential Splicing of mRNA

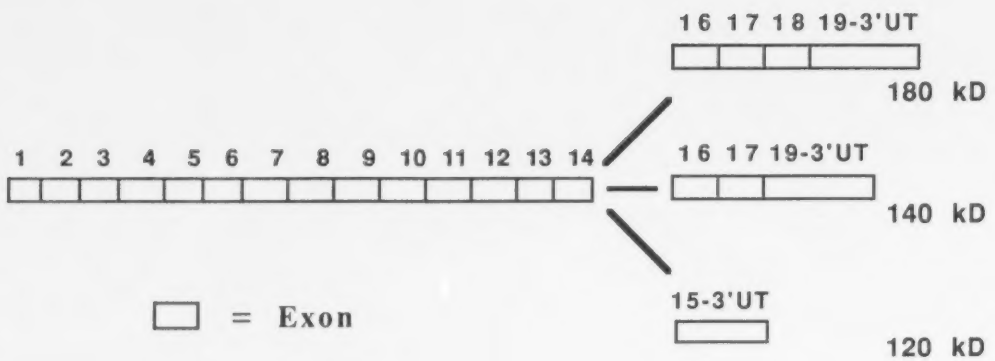


Fig. I-2. Diagramatic representation of (A) the structure of the N-CAM gene and (B) the alternative splicing that generates the mRNAs for the 180, 140, and 120 kD N-CAM polypeptides. The N-CAM gene (A) contains 19 exons, which are differentially spliced (B) to produce the three major N-CAM polypeptides. The first 14 exons are common to all of the N-CAM polypeptides. Exon 18 is present only in the 180 kD form, and exon 15 is present only in the 120 kD form. 3'UT represents the 3' untranslated region of the mRNAs.

to the 4th power of N-CAM concentration. This suggests that the developmental function of N-CAM may be modulated by variations in its prevalence. Consistent with this idea is the observation that neural crest cells decrease expression of N-CAM during migration but re-express N-CAM concomitant with their aggregation and differentiation into ganglia (Thiery et al., 1982; Edelman et al., 1983).

(ii) Changes in the N-CAM polypeptides due to alternative mRNA splicing

Another mechanism involved in regulating N-CAM-mediated adhesion is spatiotemporal variation in the N-CAM polypeptides being expressed. Immunostaining of N-CAM in the chicken embryo shows that the different N-CAM polypeptides are expressed in specific patterns during morphogenesis of individual tissues (Pollerberg et al., 1985; Daniloff et al., 1986; Moore et al., 1987). For example, the 180 kD protein is expressed only in tissues of the central and peripheral nervous system, and different regions of the brain each express varied amounts of the 180, 140, and 120 kD polypeptides during development (Chuong and Edelman, 1984; Pollerberg et al., 1985; Daniloff et al., 1986; Prieto et al., 1989). Similarly, in embryonic heart and skeletal muscle, a shift from the 140 kD to the 120 kD N-CAM polypeptide occurs concomitant with myoblast fusion (Covault et al., 1986; Wharton et al., 1989). These, and other expression patterns of the individual N-CAM proteins, suggest that the polypeptides may have different functions in development. These differences in function, however, are not well understood.

In addition to the three major N-CAM polypeptides, several additional variants of the polypeptides exist (Dickson et al., 1987; Prediger et al., 1988; Small et al., 1988; Santoni et al., 1989; Thompson et al., 1989; Small and Akeson, 1990; Reyes et al., 1991; Zorn and Krieg, 1992). These variations are due to splicing of small, extra exons into the N-CAM mRNAs, leading to the insertion of additional peptide sequences within the N-CAM proteins. The extra exons spliced into the mRNAs are present within the N-CAM gene in large introns between exons 7-8 and 12-13. Consequently, these exons are inserted into the mRNAs between the junctions of these exons (see Fig. I-3). Several different splices are known for rat, human, and frog N-CAM, but the only alternative splice known in chicken is a 93-bp insert present in skeletal muscle and heart N-CAM mRNA between exons 12-13 (Prediger et al., 1988) (see Fig. I-3).

The alternative exons are expressed in specific spatiotemporal patterns, both within given tissues and in different developing tissues. For example, in some areas of rat brain, the 30-bp VASE (or π) exon (see Fig. I-3) is present in all of the N-CAM proteins, while in other areas there is mixture of N-CAM with and without it, and in some brain regions N-CAM does not contain VASE at all (Small and Akeson, 1990). In a more extreme case, embryonic rat heart contains at least 27 different-spliced forms of N-CAM generated by alternative splicing of the VASE exon and subsets of the four exons that comprise the MSD1 insert (see Fig. I-3), in conjunction with the splicing that produces the 140 and 120 kD N-CAM polypeptides found in heart (Reyes et al., 1991). This complex splicing pattern varies during heart formation, with several of the different splicing

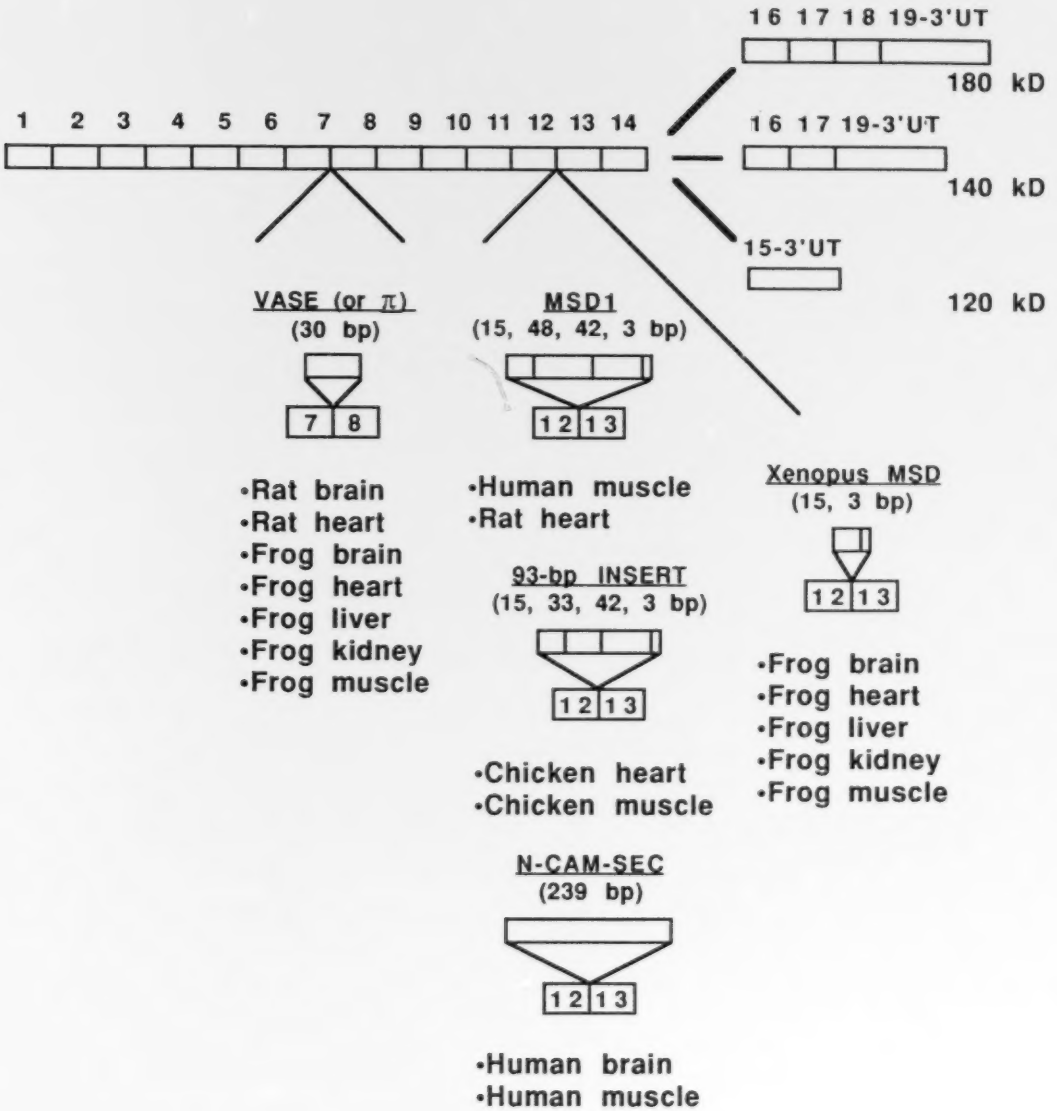


Fig. I-3. Alternative exons spliced into chicken, human, rat, and frog N-CAM mRNA at the junctions of exons 7-8 (VASE or π) and exons 12-13 (MSD1, 93-bp insert, N-CAM SEC, Xenopus MSD). Each box represents an individual exon. Boxes labelled 1-19 represent the exons that are spliced to generate the 180, 140, and 120 kD polypeptides. 3'-UT represents the 3' untranslated region of the mRNAs.

combinations being preferentially expressed at any given stage. These observations suggest that extremely fine control of N-CAM-mediated adhesion may be involved in the morphogenesis of individual tissues.

The functional significance of the alternative exons is poorly understood. Both the chick 93-bp and human MSD1 insert have a high content of proline, serine, and threonine (Prediger et al., 1988). The proline residues could introduce a hinge region that gives the N-CAM polypeptides extra flexibility and enhances binding, but this is unknown. The serine and threonine residues, however, are known to serve as sites for O-linked glycosylation in the MSD1 insert (Walsh et al., 1989). As well, the N-CAM-SEC insert (see Fig. I-3) is known to introduce an in-frame stop codon that generates a truncated form of N-CAM which is secreted (Gower et al., 1988). This secreted form of N-CAM may act as a soluble modulator of cell-cell adhesion or cell-substratum interaction as a component of the extracellular matrix (Probstmeier et al., 1989).

The mechanisms regulating the complex stage- and cell-type-specific expression of N-CAM are also poorly understood. The promoter regions for the N-CAM gene have only recently been isolated (Chen et al., 1990 - rat; Hirsch et al., 1990 - mouse; Colewell et al., 1991 - chicken). In all of these animals the N-CAM promoter does not contain a typical transcriptional start site, and transcription of the N-CAM gene instead begins at several sites that may be used differently in individual tissues (Barthels et al., 1987; Chen et al., 1990; Hirsch et al., 1990; Colewell et al., 1991; Brackenbury, 1992).

At the other end of the gene, there is evidence that alternative polyadenylation sites are also used (Goridis and Wille, 1988). At present, the function of using different start sites and polyadenylation signals is not known.

(c) Post-translational modification of the N-CAM polypeptides

In addition to the variation in N-CAM-mediated adhesion generated by alternative splicing of the major polypeptides and extra exons, a variety of post-translational modifications may also regulate N-CAM's adhesive properties. Some of the modifications, such as sulfation of N-linked carbohydrates on the proteins (Sorkin et al., 1984) and phosphorylation of amino acids in the cytoplasmic domains (Gennarini et al., 1984; Lyles et al., 1984; Sorkin et al., 1984) have unknown effects on N-CAM. However, other modifications such as the addition of N-linked carbohydrates to the N-CAM proteins (Hoffman and Edelman, 1983; Cole and Schachner, 1987; Kunemund et al., 1988) do have known effects on N-CAM-mediated adhesion.

As with the expression of the individual N-CAM proteins, glycosylation of the polypeptides is also regulated in a specific spatiotemporal manner (Cunningham et al., 1983; Crossin et al., 1984; Walsh et al., 1989; Key and Akeson, 1990, 1991). There are several potential sites for N- and O-linked carbohydrate attachment on N-CAM (Lyles et al., 1984; Barthels et al., 1987; Cunningham et al., 1987; Barton et al., 1988; Nybroe et al., 1988), but not all of these sites are used (Crossin et al., 1984; Cunningham et al., 1987). Several different N-linked carbohydrates are known to attach to these sites, including HNK-1 and polysialic acid (Rothbard et al., 1982; Kruse et

al., 1984; Key and Akeson, 1990, 1991), but only one O-linked carbohydrate is known to attach to N-CAM, this carbohydrate being associated exclusively with the MSD1 insert in human skeletal muscle (Walsh et al., 1989). It is likely that additional N- and O-linked oligosaccharides will be discovered on N-CAM, either directly on the proteins or associated with one of the alternatively spliced inserts such as MSD1.

Glycosylation of N-CAM may be involved in regulating the amount of N-CAM present at the cell surface, altering the ligand properties of N-CAM, and modulating interaction between N-CAM and other cell surface proteins. Although not proven, O-linked glycosylation may be involved in stabilizing N-CAM and increasing its cell surface expression (Walsh et al., 1989). This would be important because small variations in the surface density of N-CAM affect the rate and amount of homophilic binding (Hoffman and Edelman, 1983). Alternatively, O-linked glycosylation might influence or expand the ligand properties of N-CAM, either by modifying N-CAM's homophilic binding or by enhancing N-CAM's capacity to interact with extracellular matrix or other cell surface molecules (Walsh et al., 1989; Kadmon et al., 1990a). In the case of N-linked carbohydrates, there is direct evidence that these moieties are involved in modulating N-CAM's homophilic binding and in altering the function of other proteins that are associated with N-CAM (Hoffman and Edelman, 1983; Cole and Schachner, 1987; Kunemund et al., 1988; Rutishauser et al., 1988; Kadmon et al., 1990a, b). Of these N-linked moieties, the best characterized is polysialic acid (PSA).

PSA is a large, negatively-charged moiety that attaches to the N-CAM proteins and reduces the extent of membrane contact between apposed cells and also between individual cells and their underlying substratum (Rothbard et al., 1982; Finne et al., 1983; Acheson et al., 1991). Removal of PSA leads to an increase in the amount and intimacy of surface-surface contact and thus to enhanced interaction between membrane-associated ligands, one of which is N-CAM itself (see Fig. I-4) (Hoffman and Edelman, 1983; Rutishauser et al., 1985, 1988; Acheson and Rutishauser, 1988; Acheson et al., 1991). For example, removal of PSA increases N-CAM homophilic binding (Hoffman and Edelman, 1983) but also (a) causes a contact-dependent increase in choline acetyltransferase activity in chick sympathetic neurons (Acheson and Rutishauser, 1988), (b) augments adhesion mediated by another cell adhesion molecule, L1 (Ng-CAM), that associates with N-CAM on the cell surface (Kadmon et al., 1990a, b; Acheson et al., 1991) and, (c) increases cell-substrate interaction (Acheson et al., 1991). PSA therefore affects not only the function of N-CAM by decreasing its binding, but also the function of other ligands that may or may not be directly associated with N-CAM-mediated adhesion.

The expression of PSA on N-CAM varies during the development of individual tissues but, at the level of the whole embryo, there is more PSA present on N-CAM at younger stages and less PSA on N-CAM in older embryos (Rothbard et al., 1982; Sunshine et al., 1987). This shift in the amount of PSA is termed E (embryonic) to A (adult) conversion. The larger amount of PSA in early embryos suggests that, at this stage, the PSA may be involved in lowering

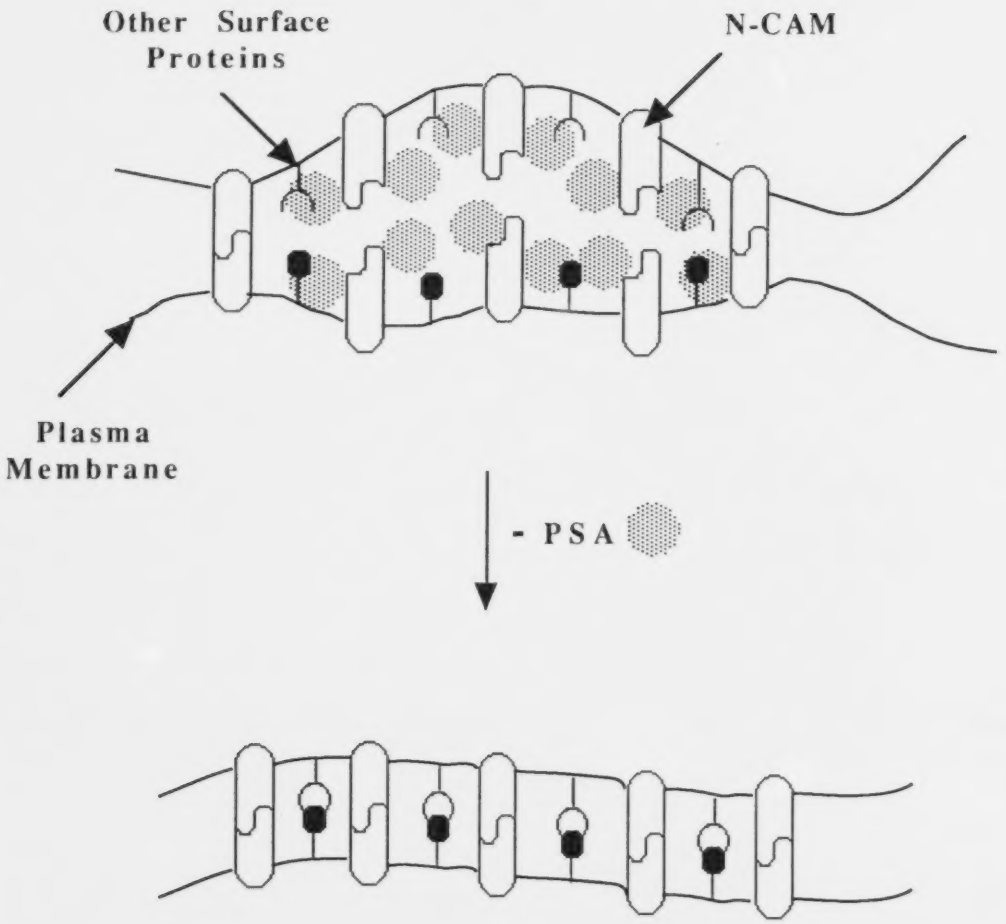


Fig. I-4. Effect of polysialic acid (PSA) on N-CAM homophilic binding and other contact-dependent events. PSA is large and negatively-charged, and prevents contact between the plasma membranes of apposed cells. Removal of PSA increases the amount and intimacy of surface-surface contact and leads to enhanced interaction between membrane-associated ligands, one of which is N-CAM.

(Modified after Rutishauser et al., 1988).

adhesion to allow for cell migration or division (Rothbard et al., 1982; Rutishauser et al., 1985). Decreases in PSA as development proceeds may then be important for increasing N-CAM-mediated adhesion to stabilize tissue architecture later in development (Hoffman and Edelman, 1983). At present, little is known of the specific function of PSA. However, in some instances, it appears that PSA may be more important in regulating cell-cell and cell-extracellular matrix (ECM) interactions than adhesion mediated by the N-CAM polypeptides per se (Acheson et al., 1991).

Role of N-CAM-mediated adhesion in development

N-CAM is thought to be involved in the formation of many embryonic tissues including brain, heart, kidney, muscle, and feather, but little is known about the specific role it plays in the morphogenesis of these structures. Two approaches are generally used to examine the function of N-CAM, namely (a) treating developing tissues with either antibodies that inhibit N-CAM homophilic binding or with an enzyme, endoneuraminidase-N, that selectively removes PSA (Buskirk et al., 1980; Fraser et al., 1984; Keilhauer et al., 1985; Rutishauser et al., 1985; Maier et al., 1986; Remsen et al., 1990; Aoki et al., 1991; Jiang and Chuong, 1992) and, (b) transfecting specific N-CAM proteins into various cell lines in order to examine the function of individual N-CAM polypeptides under particular conditions (Gower et al., 1988; Doherty et al., 1991; Lanier et al., 1991). These experiments show that N-CAM not only holds cells together but also influences a variety of other processes such as cell division (Rutishauser et al., 1985; Aoki et al., 1991), migration

(Rutishauser et al., 1985), death (Nitta et al., 1989; Lanier et al., 1991), fusion (Knudsen et al., 1990), signal transduction (Schuch et al., 1989; Doherty et al., 1991), and interaction with ECM (Cole et al., 1985; Cole and Glaser, 1986; Cole et al., 1986; Probstmeier et al., 1989, 1992; Acheson et al., 1991). It thus appears that N-CAM may have a role in modulating other primary cell functions (division, death, migration) and may be important in directing the initial formation of, and subsequent interactions between, cell populations in the embryo.

(a) Developmental role of N-CAM-mediated adhesion

The idea that cell adhesion molecules (CAMs), such as N-CAM, play a central role in morphogenesis by acting as regulators of other primary cellular processes has been termed the regulator hypothesis (Edelman, 1984a) (see Fig. I-5). During development of the embryo, morphogenetic movements bring appropriate cell populations together in a continuous set of interactions involving both cell contact and other molecular signals. The interactions of these cells at specific times and locations leads to embryonic induction, subsequent differentiation, and eventually to establishment of form. The regulator hypothesis suggests that genes for CAMs are expressed prior to the genes involved in differentiation, allowing CAMs to direct the movement of cell populations within the embryo and determine which cell populations interact with one another. By varying both the expression pattern of CAMs and their adhesive properties during morphogenesis, other primary cell processes such as migration and division may also be altered, allowing cells of different histories to be

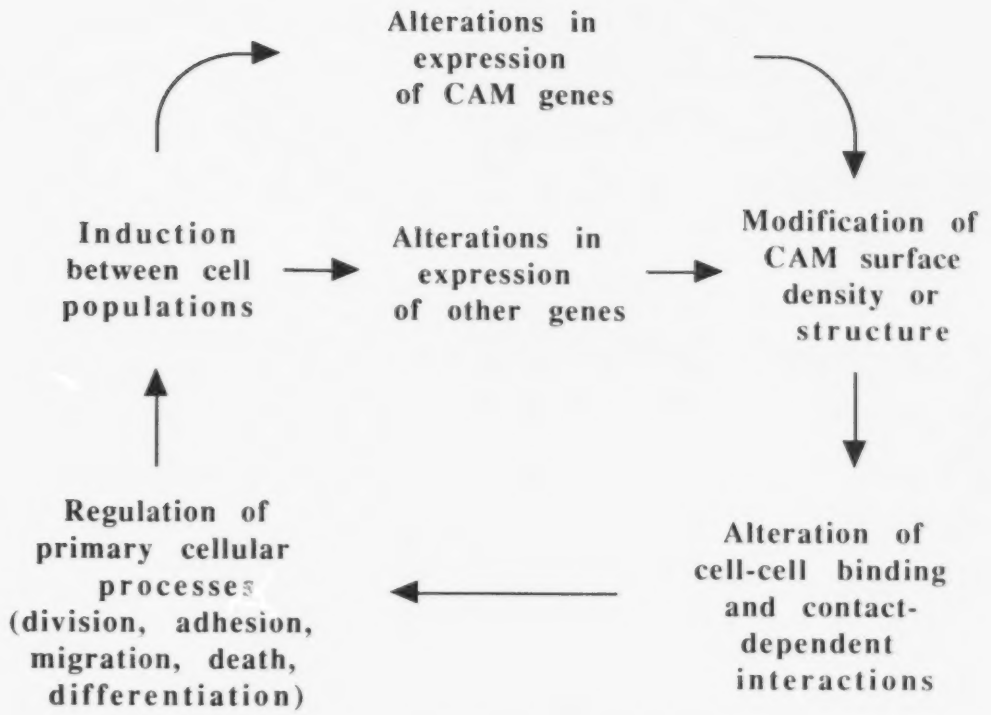

Alterations in expression of CAM genes
Modification of CAM surface density or structure
Alteration of cell-cell binding and contact-dependent interactions
Regulation of primary cellular processes (division, adhesion, migration, death, differentiation)
Induction between cell populations
Alterations in expression of other genes

Fig. I-5. Overview of the regulator hypothesis, which suggests that genes for cell adhesion molecules (CAMs) are expressed prior to genes involved in differentiation. By varying cell adhesion during development, contact between cells can be modulated, leading to changes in cell-cell interaction and in the primary cellular processes. By regulating the primary cellular processes, CAMs may be able to determine which cell populations interact during embryogenesis.

(Modified after Edelman, 1984a).

brought together and the proper sequence of interactions to occur. The increasing amount of data produced from perturbation and transfection experiments support this hypothesis by demonstrating that N-CAM-mediated adhesion is indeed interconnected to the other primary cellular processes.

A common theme underlying the studies of N-CAM function is that N-CAM mediates both cell-cell and cell-ECM interaction, these interactions are altered by modifications of the N-CAM proteins (as described above), and perturbation of these interactions causes changes in other cellular activities. Addition of PSA to N-CAM is the best understood modification, PSA reducing the extent of membrane contact between apposed cells and also between individual cells and ECM (Rutishauser et al., 1988; Acheson et al., 1991). PSA thus decreases N-CAM homophilic binding due to reduced membrane-membrane interaction (Hoffman and Edelman, 1983) but also reduces interaction between other cell surface molecules and their specific cellular or extracellular ligands (Acheson and Rutishauser, 1988; Kadmon et al., 1990a, b; Acheson et al., 1991). Keeping in mind the idea that N-CAM mediates cellular interactions and that PSA decreases them, the changes that occur upon perturbing N-CAM-mediated adhesion can be explained. For example, N-CAM mediates adhesion between fasciculating neurons in brain, and between fusing myoblast cells in muscle (Rutishauser et al., 1978; Knudsen et al., 1990). Removal of PSA from N-CAM in these tissues increases N-CAM binding and enhances neuron fasciculation and myoblast fusion, whereas antibodies to N-CAM reverse this enhancement and interfere with these events (Keilhauer et al., 1985; Knudsen et al.,

1990). Similarly, removal of PSA from N-CAM increases cell-ECM interaction, but antibodies to N-CAM interfere with this process (Cole and Glaser, 1986; Acheson et al., 1991). The decreases in cell-cell and cell-ECM interaction caused by perturbing N-CAM also affect other contact-dependent interactions and lead to additional changes in cell division, migration, death, and differentiation. For example, perturbation of normal cell-ECM interaction of fibroblastic cells with anti-N-CAM antibodies causes large increases in cell division (Aoki et al., 1991). Likewise, treatment of developing retina with anti-N-CAM antibodies and with endo-N causes changes in morphology and histology by apparently altering normal cell division and migration (Buskirk et al., 1980; Rutishauser et al., 1985). These experiments thus support the idea that N-CAM may have a regulatory role in development and may play a central role in modeling embryonic form.

(b) Developmental roles of individual N-CAM isoforms

Although the developmental function of N-CAM-mediated adhesion as a whole is becoming clearer, the roles of the individual N-CAM polypeptides remain unclear. As mentioned above, the N-CAM proteins differ in their interaction with the cell membrane; the two largest proteins are transmembrane, whereas the smallest protein is attached to the membrane via a lipid anchor. Due to these differences in structure, the proteins interact differently with the cytoskeleton and also have different mobilities within the cell membrane (Pollerberg et al., 1985, 1986). Since cell migration and sorting probably depend upon a variety of factors such as the surface

concentration of CAMs (Thiery et al., 1982), CAM flexibility at regions where the cell membrane is changing shape (Prediger et al., 1988; Knudsen et al., 1989), and interaction of CAMs with cytoskeletal components (Jaffe et al., 1990), selective expression of individual N-CAM proteins may be responsible for changing these parameters and for regulating migration and sorting. In particular, the 180 kD N-CAM protein interacts with the cytoskeleton (Pollerberg et al., 1987) and is expressed late in development, suggesting it may play a role in stabilization of tissue architecture (Pollerberg et al., 1985). Likewise, the lipid-linked 120 kD N-CAM protein tends to be expressed on cells undergoing lateral motions such as in skeletal muscle and heart (Knudsen et al., 1989; Wharton et al., 1989), perhaps due to its greater mobility and flexibility within the cell membrane (Pollerberg et al., 1986). In polarized epithelial cells, the N-CAM proteins are selectively targeted to the apical and basolateral membrane domains due to their differences in structure, suggesting an additional function of the different proteins could be to specify their own cellular localization and thus control site-specific cell-cell or cell-ECM interaction (Powell et al., 1991). Additional experiments remain to be done in order to verify these ideas and to clarify the roles of the individual N-CAM proteins in morphogenesis.

To gain insight into the function of the different proteins, it will be useful to determine which of the N-CAM molecules are restricted to particular tissues or to particular stages of development. In this way, correlations between protein expression and morphogenetic events can be established, and additional clues about protein function can be obtained. To this end, a useful system in which to

examine the expression of N-CAM during morphogenesis is feather formation. Feather development is a striking example of embryonic induction leading to the formation and patterning of multiple, discrete cell populations within a tissue. N-CAM is known to be expressed in a specific spatiotemporal pattern in developing feathers (Chuong and Edelman, 1985a, b), suggesting that N-CAM-mediated adhesion may play a role in feather morphogenesis. However, it is not known what forms of N-CAM are expressed in feathers, and little is known about the effects of perturbing N-CAM-mediated adhesion in this system. As such, feathers offer a model in which the N-CAM proteins can be characterized and their potential role in formation and patterning of cell populations examined.

Expression pattern of N-CAM in developing feathers

Chicken skin consists of two cell layers, an epidermis of ectodermal origin and dermis of mesodermal origin. Both the initial formation and subsequent development of feathers depend upon interactions between these two tissue layers; if the two layers are separated prior to the onset of feather formation, neither one will develop (Rawles, 1963). The epidermis is thought to initiate feather formation by inducing changes in the dermis, but the pattern and type of appendage formed in the skin is specified by the dermis (Sengel and Abbott, 1960; Goetinck and Abott, 1963; Rawles, 1963; Wessells, 1965; Dhouailly and Sawyer, 1984). During later stages of feather formation, continued interaction between epidermis and dermis is required for proper development of the feather filaments

(Sawyer and Fallon, 1986). Thus, by interacting with one another, the two tissue layers generate a pattern that neither tissue alone is capable of producing.

Feather development can be separated into two major phases consisting of (a) formation and patterning of the feather primordia or rudiments (embryonic days 7-9, E7-E9) and, (b) elongation of the rudiments into filaments and development of mature feathers (E10-E20) (see Fig. I-6). During the course of feather formation, N-CAM is expressed on cells involved in the induction and formation of the feather rudiments, cells that divide to form the elongating filaments, and on cells that are thought to die to create the branches of the mature feather (Chuong and Edelman, 1985a). It is therefore reasonable to conjecture that N-CAM-mediated adhesion may be involved in regulating these events and may have a role in directing feather formation. The following description of feather development is for the dorsal skin of the chicken, which is one of the earliest feather tracts to develop (Lucas and Stettenheim, 1972) and is the easiest to manipulate.

(a) Formation and patterning of the feather rudiments

Prior to the initiation of feather formation (E6), the dermis consists of a loose population of cells and is overlaid by a uniform layer of epidermal cells. No feather rudiments are present (Fig. I-6). At this stage, N-CAM is expressed at low levels on cells of dermis (Chuong and Edelman, 1985a).

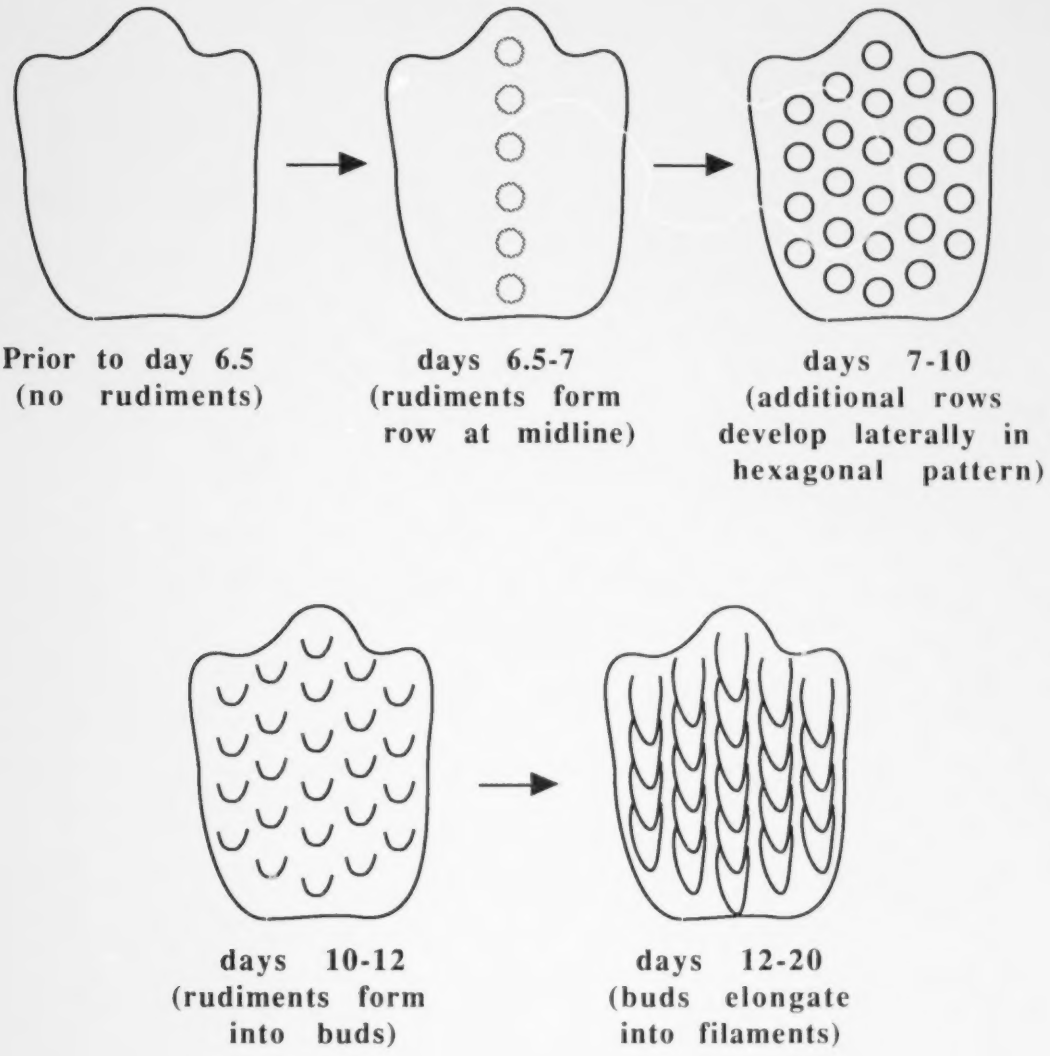


Fig. I-6. Schematic of feather formation in dorsal embryonic chicken skin, showing the time course of development.

Between E6 and E7, the epidermis induces the dermal cells to aggregate (Rawles, 1963; Sengel, 1976), and individual feather rudiments start to form at the midline of the skin (Fig. I-6). Each rudiment consists of a condensed population of dermal cells (dermal condensation) overlaid by a thickening in the epidermis (epidermal placode) (Wessells, 1965; Lucas and Stettenheim, 1972). From E7 to E9, subsequent rows of rudiments develop in a bilateral direction from the midline of the skin and are laid down in a hexagonal pattern (Davidson, 1983a, b; Mayerson and Fallon, 1985) (Fig. I-6). During this period the expression of N-CAM changes; N-CAM that was initially present at low levels throughout the dermis now becomes strongly expressed on cells that are forming the dermal condensations and decreases in expression in areas of skin that will not form feathers (Chuong and Edelman, 1985a). Within each feather rudiment the distribution of N-CAM becomes polarized and N-CAM is expressed mainly in the cephalic region of the dermal condensation. This is the region of the condensation that undergoes the least cell division as the rudiment first begins to elongate into a filament (Wessells, 1965; Desbien et al., 1991). It is therefore possible that the increased amount of N-CAM in the cephalic region may be involved in the decreased cell division in this area.

The feather rudiments now start to push above the surface of the skin due to an increase in cell division within them. Just prior to the onset of this outgrowth (E9-E10), there is a strong expression of N-CAM on cells of the epidermal placode above the center of the N-CAM-rich dermal condensation (Chuong and Edelman, 1985a). This expression of N-CAM coincides with a known inductive signal

sent from the epidermis to the dermis to begin filament formation (Lucas and Stettenheim, 1972; Sengel, 1976). If epidermis and dermis are separated at this stage, filament formation does not occur, suggesting that direct contact between the two tissue layers is required for filament outgrowth (Konig and Sawyer, 1985). It is possible that the transient, increased expression of N-CAM on both the dermal and epidermal cells may allow for direct cell-cell contact and for the exchange of signals between the two tissue layers at this time.

(b) Elongation of the rudiments into filaments and formation of mature feathers

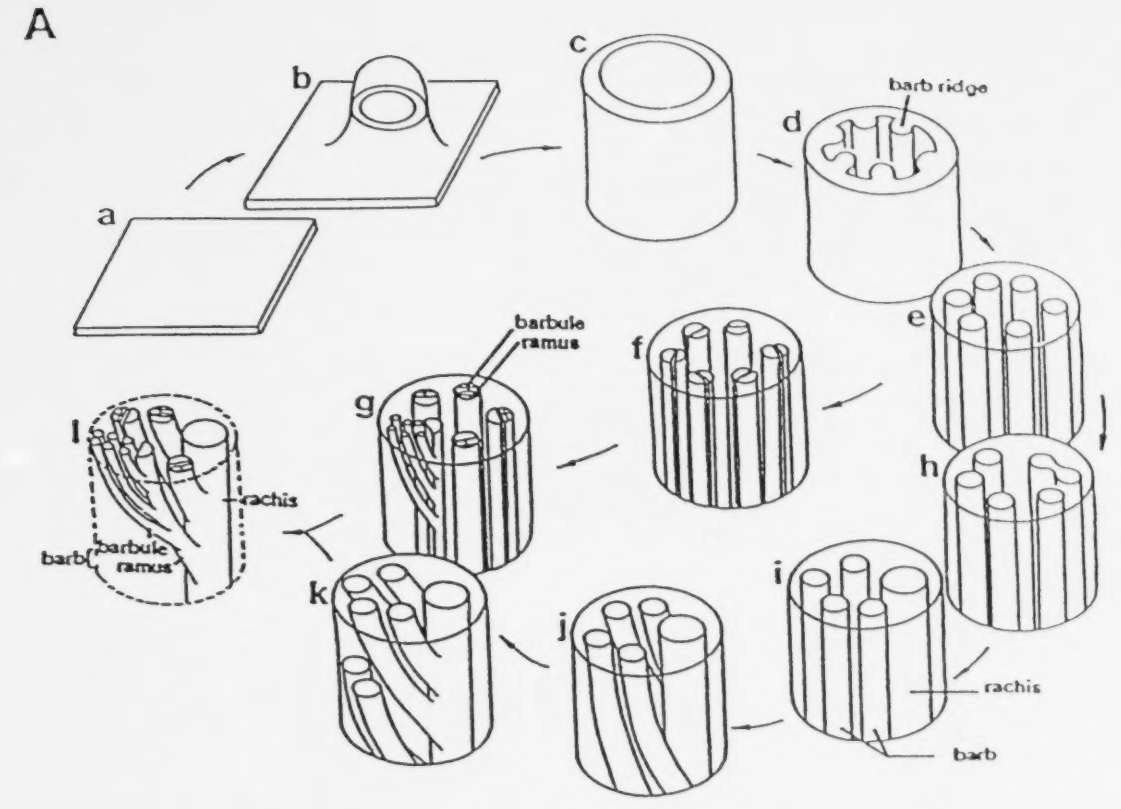
As elongation of the feather rudiments proceeds, the epidermis immediately surrounding the rudiments begins to invaginate to form a double-layered follicle (E10-E11) (Lucas and Stettenheim, 1972). The dermal condensation of the rudiment becomes encased by the walls of the ingrowing follicle and forms a dermal papilla from which the central portion (pulp, blood vessels, nerves) of the filament will arise (see Fig. I-7). Around the papilla, the epidermis thickens due to active cell division and forms a ring, called the epidermal collar, from which the inner layer of the follicle is derived. The follicle consists of a single outer layer that will form a sheath around the feather, and of an inner layer that is separated into (i) an external intermediate layer and (ii) a central basilar layer (Fig. I-7). The intermediate layer will become the major building blocks of the feather (barbs and barbules), whereas the basilar layer will form a single cell layer that separates the intermediate layer from the core

Fig. I-7. Schematic representation of filament formation and differentiation into a mature feather.

(Part A) Illustration showing how a flat layer of skin is transformed into the structure of a mature feather. Note that the number and sizes of each structure are only representative and do not reflect the actual values. (a) Flat sheet of skin. (b) Formation of feather bud. (c) A segment of the elongated bud, showing formation of an epithelial cylinder. (d and e) Invagination and cleavage of the epithelial layer into many barb ridges, which later become the individual barbs. (f and g) Segregation of each barb ridge into ramus and barbule plates. (h and i) Fusion of barb ridges into the larger rachis. (j and k) Insertion of the barbs onto the rachis. (l) A combination of processes e to g and processes e to k forms a feather structure composed of three levels of branching: tertiary branches (barbules) onto secondary branches (ramus), and secondary branches onto a primary branch (rachis).

(Part B) Feather structures are shown with appropriate terminology. (a) Longitudinal section of a developing filament. Cross sections at the levels of the two lines indicated on the longitudinal secton are shown to the right. (b) Structure of a bilaterally symmetrical adult feather.

(Taken from Chuong and Edelman, 1985a).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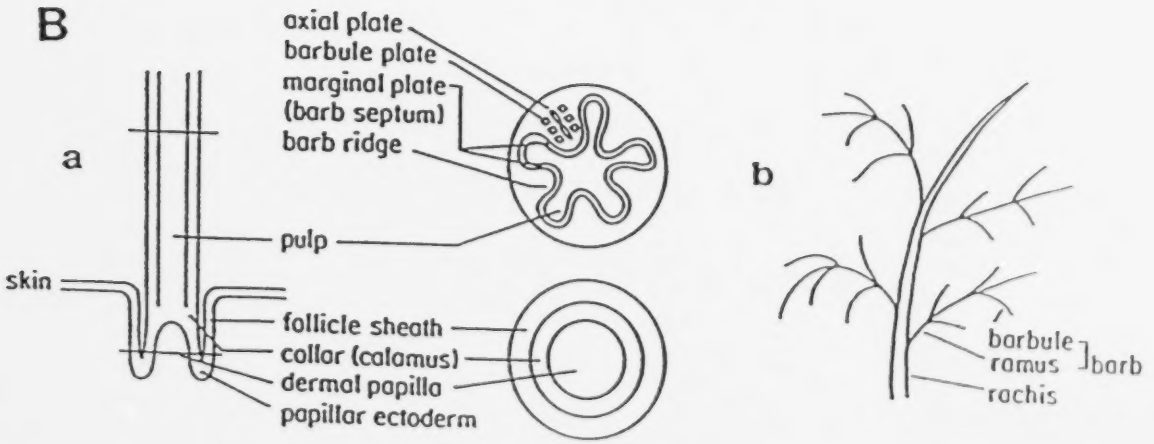
B
a
axial plate
barbule plate
marginal plate
(barb septum)
barb ridge
pulp
skin
follicle sheath
collar (calamus)
dermal papilla
papillar ectoderm
b
barbule
ramus
barb
rachis

of dermal cells (pulp). At the base of each follicle, erector muscles are becoming organized in the surrounding dermis. These muscles may reach the wall of the follicle but do not attach to it at this time. Throughout this developmental period, N-CAM is expressed on cells that will form the dermal papilla, epidermal collar, and the muscles of the feather (Chuong and Edelman, 1985a).

Barb ridges now begin to arise from the intermediate layer of the follicle by rearrangement of cells into a series of ridges that are parallel to the long axis of the filament (Lucas and Stettenheim, 1972) (Fig. I-7). Nine to eleven ridges develop around the circumference and lengthen towards the base of the filament. The internal basilar layer follows the contours of these ridges and forms into marginal plates (barb septa). Within each barb ridge, the cells reorganize into two rows called barbule plates (Fig. I-7). The barbule plates are separated from one another by a layer of cells called the axial plate. The cells of the barbule plates will form two structures: the cells closest to the central pulp will become a major branch of the feather (ramus), whereas the more lateral cells will become minor branches (barbules). Cells of the axial plate degenerate to form two rows of barbules, which subsequently attach onto the central ramus (Fig. I-7). Cells of the marginal plate also degenerate to create individual barbs, each barb containing a ramus and the two rows of barbules. Several of the barbs fuse to form the main stem (rachis) of the feather. The remaining barbs attach to the rachis in a similar fashion to which the rows of barbules attached to the ramus. In this manner the final branched structure of the feather is formed. During this process (E12-E16), N-CAM is expressed on the cells of the axial

and marginal plates that degenerate to create the spaces between the barbules and the barbs (Chuong and Edelman, 1985a). It is thought that these cells may undergo programmed death, suggesting that the expression of N-CAM on these cells may be involved in determining their fate and thus in creating the final structure of the feather.

The remainder of feather development (E16-E20) consists of additional elongation of the filament, resorption of the pulp, and keratinization of the barbs (Lucas and Stettenheim, 1972; Haake et al., 1984). The filament becomes primarily acellular, except at its base, where N-CAM continues to be expressed on cells of the dermal papilla, epidermal collar, and the muscle cells around the follicle. Shortly after hatching, the sheath around each filament dries and flakes away and the individual barbs are released (Lucas and Stettenheim, 1972). As well, the erector muscles attach to the base of each filament to allow for the movement of the individual feathers (Lucas and Stettenheim, 1972).

Experimental Objectives

When the expression pattern of N-CAM in developing feathers is placed into the context of the regulator hypothesis, it is clear that N-CAM-mediated adhesion may be involved in directing feather morphogenesis by regulating epidermal-dermal interaction, cell division, and perhaps even cell death. At present, neither the forms of the N-CAM polypeptides nor the expression pattern of PSA have been examined in detail in developing feathers. As well, only preliminary investigations into the function of N-CAM-mediated adhesion in developing feathers have been done (Jiang and Chuong,

1992). The goals of this thesis were therefore to (a) characterize the various molecular forms of N-CAM present in developing feathers and (b) determine in greater detail what changes in feather development occur upon perturbing N-CAM-mediated adhesion in this system. By correlating changes in morphogenesis with perturbation of N-CAM-mediated adhesion, information about the role of N-CAM in developing feathers should be obtained.

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CHAPTER II

Structural Variants of the Neural Cell Adhesion Molecule (N-CAM) in Developing Feathers[1]

Introduction

Cellular adhesion is mediated by proteins called cell adhesion molecules (CAMs), which bind to one another and promote membrane contact between cells (Edelman, 1983). The neural cell adhesion molecule (N-CAM), one of the best characterized CAMs, mediates calcium-independent, homophilic adhesion between many different cell types in the developing embryo (Hoffman et al., 1982; Hall et al., 1990). N-CAM is expressed in specific, regulated patterns in many tissues during embryonic development (Edelman, 1983; Chuong and Edelman, 1985a; Crossin et al., 1985). In many cases, these patterns of expression are coincident with interactive, or inductive, events between cell groups or tissues, for example during kidney development, formation of the nervous system, and formation of the feather (Thiery et al., 1982; Edelman, 1983; Chuong and Edelman, 1985a, b). This implies that control of N-CAM-mediated cellular adhesion may be important in the generation of embryonic form.

There are several ways in which the adhesiveness mediated by N-CAM may be regulated during embryonic development, namely, (a) changes in the amount of N-CAM being expressed (Rutishauser et al., 1978), (b) changes in the structure of N-CAM polypeptides being

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expressed (Pollerberg et al., 1985; Murray et al., 1986) and (c) modification of the N-CAM polypeptides by addition of carbohydrate moieties (Hoffman and Edelman, 1983; Walsh et al., 1989). In embryonic chicken brain, N-CAM exists as at least three protein forms (M_r = 180, 140, and 120 kD) that are encoded by different mRNAs produced from a single gene (Murray et al., 1986). The selective expression of the N-CAM polypeptides on different cell types during development (Pollerberg et al., 1985; Daniloff et al., 1986; Moore et al., 1987) suggests that the different polypeptides may have different functions in morphogenesis. Recently, several additional variants of the N-CAM polypeptides have been identified (Dickson et al., 1987; Prediger et al., 1988; Small et al., 1988; Santoni et al., 1989; Thompson et al., 1989; Small and Akeson, 1990; Reyes et al., 1991; Zorn and Krieg, 1992). These variations, which are due to insertion of small additional peptide sequences within the protein, may serve as attachment sites for carbohydrate moieties that are also involved in tissue-specific regulation of N-CAM-mediated cell-cell adhesion (Walsh et al., 1989).

One carbohydrate moiety that alters the adhesive function of N-CAM is polysialic acid (PSA) (Rothbard et al., 1982; Hoffman and Edelman, 1983; Rutishauser et al., 1988). PSA is large and negatively charged, and it inhibits N-CAM-mediated adhesion by preventing membrane contact between apposed cells (Hoffman and Edelman, 1983; Rutishauser et al., 1988; Acheson et al., 1991). During development of the embryo, the amount of PSA attached to N-CAM varies (Rothbard et al., 1982; Sunshine et al., 1987). Decreases in PSA may be important for increasing N-CAM-mediated adhesion to stabilize

tissue architecture late in development (Hoffman and Edelman, 1983). Alternatively, increased amounts of PSA may be involved in lowering adhesion between cells to allow for cell migration or division (Rothbard et al., 1982; Rutishauser et al., 1985). At present, however, little is known of the specific function of PSA, and of the different N-CAM polypeptides.

Feather development in embryonic chicken offers a model system to examine the function of N-CAM and PSA in the formation and patterning of discrete cell populations within a tissue. During feather development, cells in the dermis proliferate and condense into a defined pattern of cell groups (Davidson, 1983a). These groups, called dermal condensations, are overlain by thickenings in the epidermis called epidermal placodes, which together with the condensations constitute the individual feather rudiments (Davidson, 1983a; Mayerson and Fallon, 1985). Within each rudiment, the dermal and epidermal cells interact, proliferate, and develop into a feather bud (Sengel, 1976; Sawyer and Fallon, 1983). This bud elongates due to cell proliferation, and develops into a feather filament (Wessells, 1965; Lucas and Stettenheim, 1972). Cells within the feather filament rearrange into a series of ridges that are parallel to the long axis of the filament (Lucas and Stettenheim, 1972). These ridges develop into the branches, called barbs and barbules, of the mature feather.

During the development of feathers, N-CAM is expressed on some of the cells that are involved in formation of the feather rudiments and feather filaments (Chuong and Edelman, 1985a). Specifically, N-CAM is expressed on cells that form the dermal condensations,

epidermal cells from which the elongating feather filament arises, and on epidermal cells that are thought to die in the filaments to generate spaces between the barbules. Thus, during feather development, N-CAM is expressed on cells that are actively dividing, and may be expressed on cells undergoing death. It is possible that N-CAM-mediated cellular adhesion may be involved in regulating these events and in directing feather formation. At present, however, the function of N-CAM in developing feathers is unknown.

As a first step towards determining how N-CAM functions in feather development, we have analyzed the structure of N-CAM in the feather. Our results indicate that the function of N-CAM may be modulated by variations in the amount of the protein, variations in the structure of the polypeptide chain, and variations in the N-linked carbohydrates and PSA attached to the N-CAM.

Materials and Methods

Materials - Reagents were purchased from the sources indicated: BioBeads SM-2, rabbit anti-mouse IgM HRP, and materials for SDS-PAGE (Bio-Rad); restriction enzymes (BRL); AMV reverse transcriptase and endoglycosidase-F (Boehringer Mannheim); Mowiol 4-88 (Calbiochem); goat and rabbit serum (Gibco); FITC- and RITC-conjugated secondary antibodies (ICN Immunobiologicals); goat anti-rabbit IgG alkaline phosphatase (Jackson Immunoresearch); Tissue-Tek O.C.T. compound (Miles Scientific); HA Filter (Millipore); 3,3' diaminobenzidine tetrahydrochloride (Polysciences); cesium trifluoroacetate, dNTPs, and Sepharose CL-4B (Pharmacia). All other reagents were obtained from BDH or Sigma.

Fertile White Leghorn eggs were obtained from the University of Alberta Experimental Farm, Poultry Division. Eggs were incubated at 37°C, 55% relative humidity, for up to 18 days. Embryos were staged according to the method of Hamburger and Hamilton (1951). E6 to E18 denote embryonic age from 6- to 18-days after the onset of incubation.

Tris buffered saline (TBS) is 50mM Tris-HCl, 150mM NaCl, pH 8.0. TTBS is TBS, 0.1% Tween 20. Chromogen diluent is TBS, 10mM $MgCl_2$. Phosphate buffered saline (PBS) was made as follows: 8g of NaCl, 0.2g of KCl, 0.2g of KH_2PO_4, 0.15g of Na_2HPO_4/liter (pH 7.4) (Dulbecco and Vogt, 1954).

Antibodies - Rabbit polyclonal and mouse monoclonal antibodies (mAbs) were used as prepared by Hoffman et al. (1982) (anti-N-CAM CAM-6 and 802) and Watanabe et al. (1986) (anti-N-CAM 5e), Dodd et al. (1988) (anti-PSA 5A5), Gallin et al. (1983) (anti-L-CAM 7D6), Lin et al. (1985) (anti-tropomyosin CH1), and Skalli et al. (1986) (anti-α–actin αsm-1). Anti-N-CAM mAbs CAM6 and 5e, and anti-N-CAM polyclonal antibody 802 all react with protein epitopes. All three anti-N-CAM antibodies recognize the 180, 140, and 120 kD polypeptides in embryonic chick brain and do not react with other proteins, indicating that any carbohydrates recognized by anti-N-CAM 802 are unique to N-CAM. All other antibodies listed are monoclonal. An irrelevant mAb against chick liver bile cannaliculi, culture supernatant from SP2/0 myeloma cells, and rabbit or goat serum were used as controls. Anti-α–actin antibody

was purchased from Sigma. Anti-tropomyosin CH1, anti-PSA 5A5, and anti-N-CAM 5e antibodies were from the Developmental Studies Hybridoma Bank (Baltimore, MD).

Immunoperoxidase staining of wholemounts - Skin was removed from stage 29-44 (E6-E18) embryos by blunt dissection. For separation of epidermis and dermis (stage 29-35, E6-E9), skin was mounted epidermal-side down on HA filter, incubated in 2x Tyrode's solution with EDTA (Konig and Sawyer, 1985) for 20 min, and the dermis was peeled off with forceps and mounted on another filter. Separated epidermis and dermis were fixed in 4% paraformaldehyde with 3% hydrogen peroxide overnight at 4°C. Whole-skin samples were fixed on ice for 2 h in a freshly-made solution of 20% dimethylsulfoxide (DMSO) in methanol. Hydrogen peroxide was added to a concentration of 3%, and the sample was left overnight at 4°C. The following day, whole-skin samples were soaked in methanol for several hours to remove excess DMSO. All samples were then washed 2x10 min in TBS and incubated in primary antibody overnight (rabbit antibodies (10 mg/ml) and mouse ascites were diluted 1:900 in 20% goat serum or 20% rabbit serum in TBS, respectively; for mouse mAb culture supernatants, rabbit serum was added to 20% final concentration). Samples were washed 5x1 h in TBS, and then incubated in horseradish peroxidase-conjugated secondary antibody against the appropriate species (secondary antibody was diluted 1:300 in 20% goat or rabbit serum in TBS, accordingly). Samples were washed as above, and the peroxidase was localized using 3,3' diaminobenzidine tetrahydrochloride (DAB) (0.05%) and hydrogen peroxide (0.02%) in TBS. After DAB treatment, samples were washed

several times in TBS, dehydrated through a graded alcohol series, soaked in xylene for several minutes, and mounted on a glass slide with Permount. Wholemounts were photographed on a Zeiss Axiophot microscope using Kodak Tri-X 400 film.

Immunofluorescent staining - Stage 29-35 (E6-E9) embryos and dorsal skins from stage 36-44 (E10-E18) embryos were fixed in 4% paraformaldehyde overnight at 4°C, cryoprotected by immersion in a graded sucrose series, and mounted in Tissue-Tek O.C.T. compound. 10 µm frozen sections were cut on a cryostat, collected on chrome-alum subbed slides, and stored at -80°C. Prior to staining, sections were pre-incubated in 0.7% carrageenan, 0.1% Triton X-100, in TBS (Sofroniew and Schrell, 1982) for 1 h to block non-specific staining. Sections were then incubated overnight with primary antibody (polyclonal antibodies and monoclonal ascites were diluted 1:400 in blocking solution, and mAb culture supernatants were used undiluted). After 5x10 min washes in TBS, sections were incubated with FITC- and RITC-labelled secondary antibodies against the appropriate species (1:200 in carrageenan blocking solution for anti-rabbit antibodies, 1:100 for anti-mouse antibodies) for 2 h, washed five times again, and mounted in Mowiol (Osborn and Webber, 1982), modified by the addition of 2,4-diazabicyclo (2,2,2) octane (Zalik et al., 1990). Sections were photographed on a Zeiss Axiophot microscope using Kodak Tri-X 400 film.

Immunoaffinity Purification of N-CAM - N-CAM was purified using a specific mAb (CAM-6) coupled to Sepharose CL-4B as described previously (Hoffman et al., 1982).

Tissue Extracts, PSA Removal, and Endo-F Digestion - Tissue was homogenized using a Polytron homogenizer (Brinkmann) in 0.5% CHAPS in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 5 µg/ml DNase, and 1 mM PMSF. Samples were kept on ice for 10 min with occasional vortexing, and cleared by centrifuging 10 min in a microfuge at 4°C. Samples were mixed with SDS-PAGE sample buffer and heated 1 min in a boiling water bath. For PSA removal, samples were heated for 30 min in a boiling water bath or digested with *Clostridium perfringens* neuraminidase as described by Hoffman et al. (1982). For removal of N-linked carbohydrate, desialylated N-CAM was incubated 48 h at 37°C in 20 mM potassium phosphate, pH 7.2, 50 mM EDTA, 0.05% sodium azide, 2% n-octyl-glucoside, and 0.5 U endoglycosidase-F (endo-F) in a volume of 50 µl. To determine the number of N-linked carbohydrate attachment sites on N-CAM, samples were treated with endo-F as above except that 1:4 serial dilutions of enzyme were used to sequentially digest the sugar chains from the protein (Elder and Alexander, 1982; Crossin et al., 1984). For storage, samples were frozen in a dry ice-ethanol bath and kept at -80°C.

Immunoblots - Approximately 200 µg of total protein or 30 µg affinity-purified N-CAM was fractionated by SDS-PAGE (Laemmli, 1970) on 4% gels and transferred to nitrocellulose (Towbin et al., 1979). Non-specific binding was blocked by incubation in TBS-5% BSA for 2 h. Primary antibody, wheat germ agglutinin-HRP (WGA-HRP) conjugate, or biotinylated peanut (PNA) or jackfruit (jacalin) lectin (Vector Laboratories) diluted to 10 µg/ml in TBS-1% BSA was incubated with the blot overnight. Blots were washed 2x10 min in

TTBS and 3x10 min in TBS. For biotinylated-lectin blots, streptavadin-alkaline phosphatase conjugate (1:5000 in TBS-1% BSA) was added for 1 h, the blot was washed as above, and developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT). For WGA-HRP blots, the blot was developed directly using 4-chloro-1-napthol and hydrogen peroxide. For all lectin blots, samples were pre-digested with neuraminidase to expose terminal sugar residues and enhance lectin binding (Russell et al., 1984). All other blots were incubated with an alkaline phosphatase- or HRP-conjugated secondary antibody (diluted 1:15000 or 1:3000 in TBS-1% BSA, respectively), washed as above, and visualized using BCIP and NBT (for alkaline phosphatase) or 4-chloro-1-napthol and hydrogen peroxide (for HRP) as substrates.

Northern Blot Analysis - Total cytoplasmic RNA was prepared using the cesium trifluoroacetate method (Okayama et al., 1987). 5 μg were fractionated by electrophoresis on 0.5% agarose gels in the presence of formaldehyde and transferred to nitrocellulose (Maniatis et al., 1982). Blots were hybridized overnight at 55°C with a ^{32}P-dCTP-labelled (New England Nuclear, 3000Ci/mmol) DNA probe prepared with a random primers labelling kit (BRL) according to manufacturer's instructions, using a 1210-bp PstI fragment from pEC208 (Hemperly et al., 1986a) as a template. Blots were washed to 0.1 x SSC at 65°C and exposed to RX X-ray film (Fuji) at -80°C.

cDNA Synthesis and PCR - Oligonucleotides with the following sequences were synthesized by Dr. K. Roy (Department of Microbiology, University of Alberta): Oligo 1 (TGTTTTTTCTCGG-AGCCGCA); Oligo 6 (AGCAGAGTACATCTGCATCG); Oligo 8 (TTGAGA-

GTCAGGGACGATAC); Oligo 11 (GGTGCCCATCCTCAAATACA); Oligo 13 (ATGGAGTTTCCGTCTTCTCC); Oligo 19 (CAGATTTGTCTTCTACTGGG); Oligo 3' (TCCCATTCTCACTGGTGTAA). Oligos 1, 6, 11, and 3' are identical to the sense strand of N-CAM cDNA. Oligos 1, 6, and 11 hybridize to the antisense strand approximately 30-bp 5' of the splice junctions between exons 1-2, exons 6-7, and exons 11-12, respectively. Oligo 3' hybridizes to the antisense strand 118-bp upstream of the poly-A tail. Oligos 8, 13, and 19 are antisense, and hybridize to N-CAM mRNA about 30-bp 3' of the splice junctions between exons 7-8, exons 12-13, and exons 17-19 (in 140 kD N-CAM) or 18-19 (in 180 kD N-CAM) (Owens et al., 1987) (see Fig. II-6A). All of the N-CAM sequences were obtained from GenBank, release 61. Two additional primers, pBA9 (GACTCGAGTCGACATC-GAT_{17}) and pBA10 (GACTCGAGTCGACATCG) (gifts from Mr. Phillip Barker, Department of Cell Biology and Anatomy, University of Alberta) were used for specific first strand cDNA synthesis (pBA9) (see below) and for RACE PCR (pBA10) (Frohman et al., 1988) in conjunction with Oligo 3'.

Total cellular RNA (isolated as above) was used as a template for first strand cDNA synthesis. Briefly, 15 µg of total RNA was coprecipitated with 300 ng of random primers, oligo-dT, or primer pBA9, dried, and dissolved in 10 µl annealing buffer (250 mM KCl, 10 mM Tris, pH 8.3, at 50°C). The mixture was denatured for 10 min at 65°C and allowed to cool to room temperature. Then 16.5 µl of reverse transcriptase mix (24 mM Tris, pH 8.3 at 50°C, 16 mM $MgCl_2$, 8 mM DTT, 2 mM each dNTP, 10 U RNasin, 20 U AMV reverse transcriptase) was added to each annealing reaction, and incubated at

50°C for 1 h. 2 µl of this reaction mix was used directly as a template for amplification by PCR under the following conditions in a 100 µl volume: 50 mM KCl, 10 mM Tris, pH 9.0, 0.01% gelatin, 200 µM each dNTP, 1.5 mM $MgCl_2$, 40 pmol each primer, and 2.5 U Taq polymerase (Promega). Samples were taken through 30 PCR cycles as described previously (Small and Akeson, 1990). 20 µl aliquots were loaded on agarose gels and visualized by post-staining for 30 min with 0.75 µg/ml ethidium bromide. Gels were Southern blotted onto Genescreen Plus (Dupont), and probed with a ^{32}P-dCTP-labelled DNA probe prepared using the 3556-bp EcoR1 insert of pEC208 (Hemperly et al., 1986a) as a template, as described above.

Results

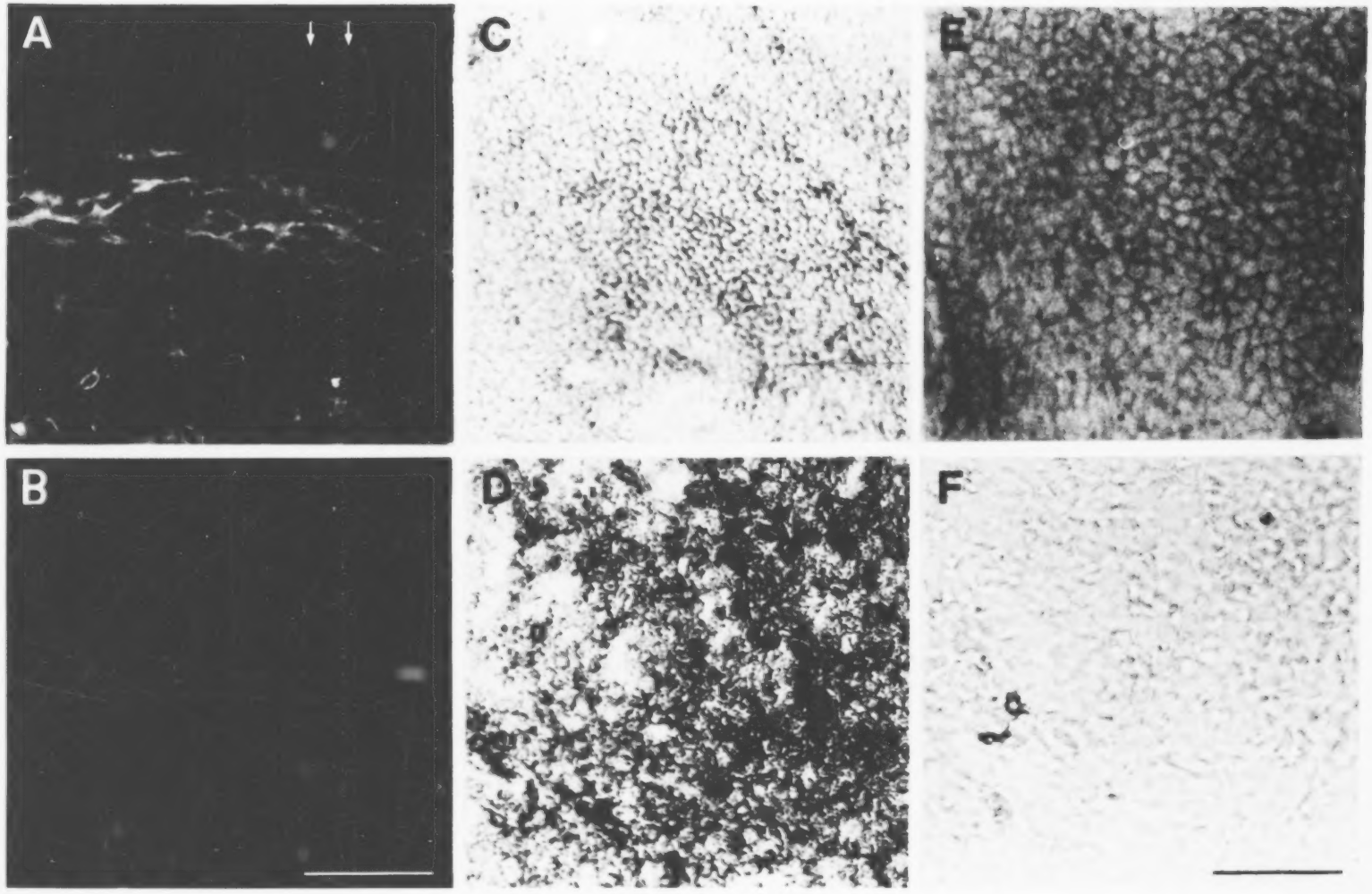
The terminology we use in describing feather structures and the processes involved in feather formation is as outlined by Chuong and Edelman (1985a) and Lucas and Stettenheim (1972).

Distribution of N-CAM and PSA in Developing Feathers

We examined the expression of N-CAM and PSA in developing feather rudiments from stage 28 (E6) to stage 44 (E18) to determine the expression of these molecules prior to (stage 28-29) and during (stage 30-44) feather development.

Prior to the formation of discernible feather structures in the dorsal skin, we found that N-CAM was expressed at low levels on cells of the epidermis and dermis at stage 29 (E6). As shown in Fig. II-1A, both epidermal and dermal cells were N-CAM-positive. Wholemounts of separated epidermis (Fig. II-1C) and dermis

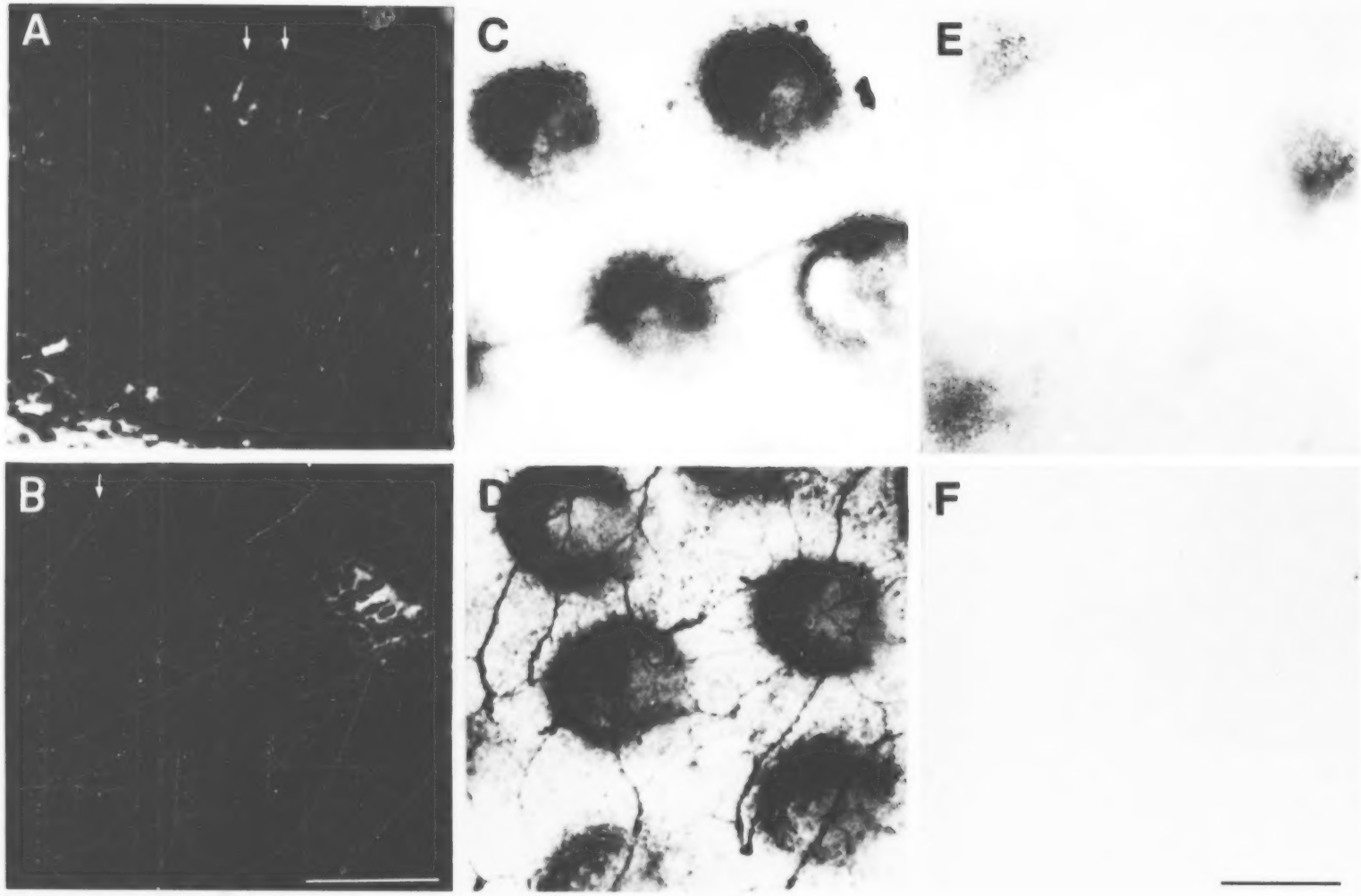
Figure II-1. Immunofluorescent staining of embryonic skin sections (A-B) and immunoperoxidase staining of skin wholemounts (C-F) at stage 29 (E6) with anti-N-CAM CAM6 mAb (A, C, D), anti-PSA 5A5 mAb (B), anti-L-CAM 7D6 mAb (E), or an irrelevant mAb (F). (A) N-CAM is expressed throughout both epidermis and dermis as determined by staining with anti-N-CAM mAb. Arrows denote upper boundary of the epidermis. (B) PSA is not expressed in either epidermis or dermis at this stage. (C) Separated epidermis stained with anti-N-CAM mAb, showing uniform distribution of N-CAM. (D) Separated dermis stained with anti-N-CAM mAb, showing scattered distribution of dermal N-CAM. (E) Separated epidermis stained with anti-L-CAM mAb to verify N-CAM epidermal staining has the same pattern as L-CAM staining. (F) Whole-skin stained with irrelevant mAb against bile cannaliculi. Scale bars = 50 μm.


A
B
C
D
E
F

(Fig. II-1D) likewise showed that N-CAM was present throughout both tissue layers. Examination of epidermal N-CAM staining at high magnification showed that N-CAM was expressed over the entire surface of the epidermal cells (data not shown). The specificity of N-CAM staining was demonstrated by staining with an irrelevant mAb (Fig. II-1F). As well, several different anti-N-CAM antibodies (monoclonal and polyclonal) gave the same staining pattern (data not shown). L-CAM is present only on epidermal cells in developing feathers (Chuong and Edelman, 1985a), so we verified that N-CAM staining in the epidermis was in the same pattern as L-CAM staining (Fig. II-1E). L-CAM staining in the epidermis showed the same pattern of expression for all ages of skin examined (data not shown). PSA was not detectable in either epidermis or dermis at this stage (Fig. II-1B).

During the formation of feather rudiments (stage 30-35, E7-E9), N-CAM expression changed from being distributed throughout the epidermis and dermis (as in Fig. II-1) to a focused distribution on cells of the developing epidermal placodes and dermal condensations (Fig. II-2A, C, and E). Specifically, N-CAM that was initially present on a loose collection of dermal cells became localized predominantly to dermal cells forming the condensations (Fig. II-2, A and C). Our findings, in conjunction with previous observations (Chuong and Edelman, 1985a), showed that N-CAM expression in the epidermis changed in a similar fashion; we found that from stage 30-34 (E7-E8) N-CAM was present throughout the epidermis (identical to Fig. II-1, A and C) but became localized to cells of the epidermal placodes by

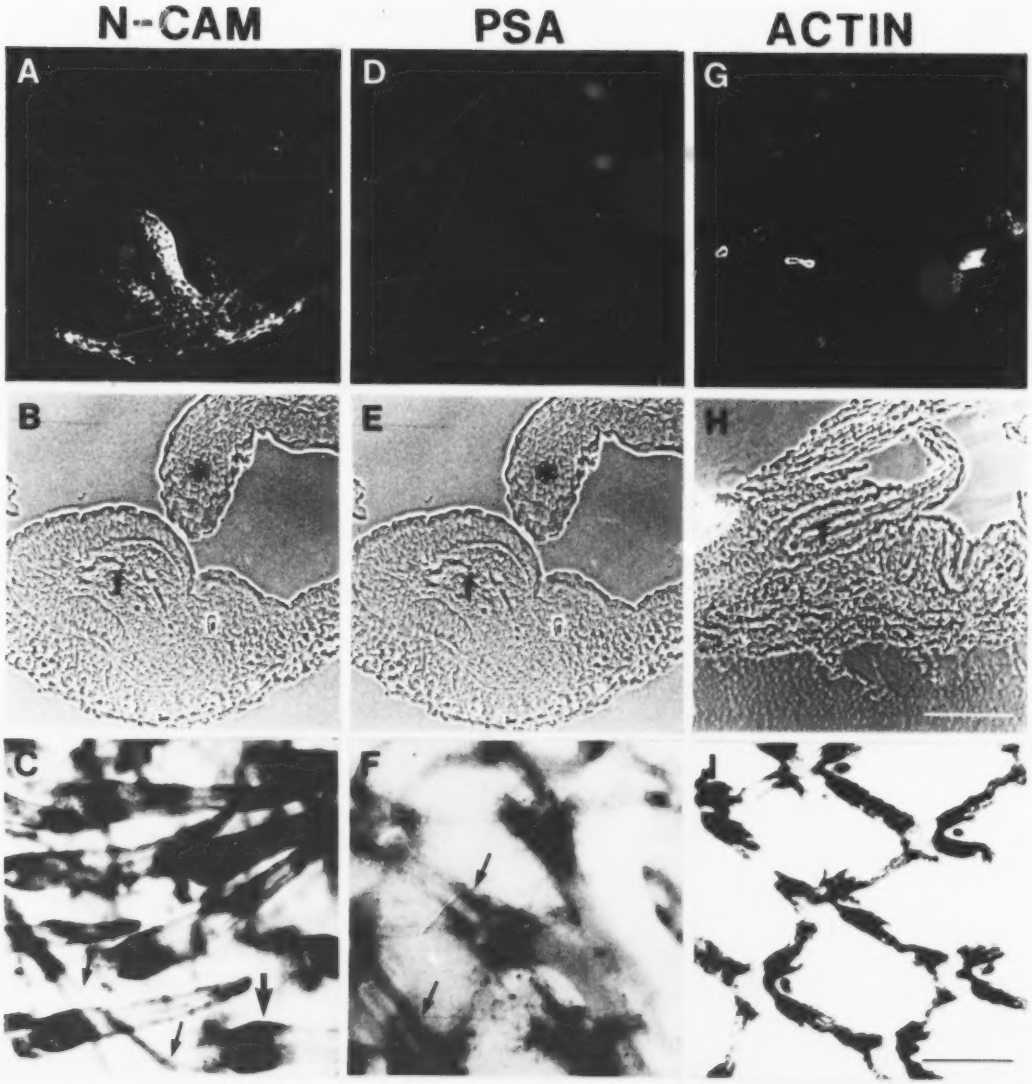
Figure II-2. Immunofluorescent staining of skin sections (A-B) and immunoperoxidase staining of skin dermal (C-D) or epidermal (E-F) wholemounts at stage 35 (E9) with anti-N-CAM CAM6 mAb (A, C, E) or anti-PSA 5A5 mAb (B, D, F). At this stage the expression of N-CAM is now localized to the dermal condensations (A & C) and epidermal placodes (A & E) of the feather rudiments. Note that Fig. II-2A represents N-CAM staining in a single condensation; localization of N-CAM to the dermal condensations is shown by the absence of staining in the surrounding dermis in Fig. II-2C. Arrows indicate upper margin of epidermis. PSA is first expressed in the dermis (B & D) at this stage, but is not present in the epidermis at this (B & F), or any other stage we examined. Skins are oriented with cephalic to the left and caudal to the right. Scale bars = 50 μm (A-B) and 200 μm (C-F).


A
B
C
D
E
F

stage 35 (E9) (Fig. II-2, A and E). It was at stage 35 (E9), when N-CAM was strongly expressed by cells of the epidermal placode and dermal condensation, that we first detected PSA in the dermal condensations (Fig. II-2, B and D). Both N-CAM and PSA were localized predominantly to the cephalic side of the condensation (Fig. II-2, C and D). PSA was not present in the epidermis at this stage (Fig. II-2F), nor was it detected in this tissue layer at any stage of feather development examined.

During the remainder of feather development (stage 36-44, E10-E18), the epidermis invaginates to form a double-layered follicle and the feather rudiments elongate into filaments (Lucas and Stettenheim, 1972). We found that N-CAM was expressed in the elongating feather filaments as previously described (Chuong and Edelman, 1985a) and was also present on cells at the base of the filaments (Fig. II-3, A and C). Staining with an anti-α-actin antibody specific for smooth muscle (Fig. II-3, G and I) and with an anti-tropomyosin antibody that recognized skeletal muscle (data not shown) demonstrated that these cells were smooth muscle. As filament formation progressed, N-CAM was expressed more strongly on the smooth muscle cells and the staining appeared identical to that in Fig. II-3I (data not shown). PSA was expressed in the dermal condensations until stage 36 (E10) (as in Fig. II-2, B and D) and was then localized to the smooth muscle cells for all subsequent stages of feather formation examined (stage 37-44, E11-E18) (Fig. II-3, D and F). As with N-CAM, the expression pattern of PSA shifted from being

Figure II-3. Immunofluorescent staining of skin sections (A, D, G), phase contrast photographs of the same fields (B, E, H), and immunoperoxidase staining of whole-skins (C, F, I) at stage 38 (E12). Staining is with anti-N-CAM CAM6 (A-C), anti-PSA 5A5 (D-F), or anti-α-actin αsm-1 mAb (G-I). N-CAM is expressed in both the filaments (f) (* is an oblique section of a filament) and in the underlying dermis both within the filament (large arrows) and on developing smooth muscle (small arrows) (A & C). PSA is expressed only on cells in the dermis that are forming smooth muscle (small arrows) (D & F). Staining with anti-α-actin mAb (G & I) shows the dermal smooth muscle cells that will become the erector muscles of the feathers. Scale bars = 200 μm (C, F and I) and 50 μm for all other photographs.

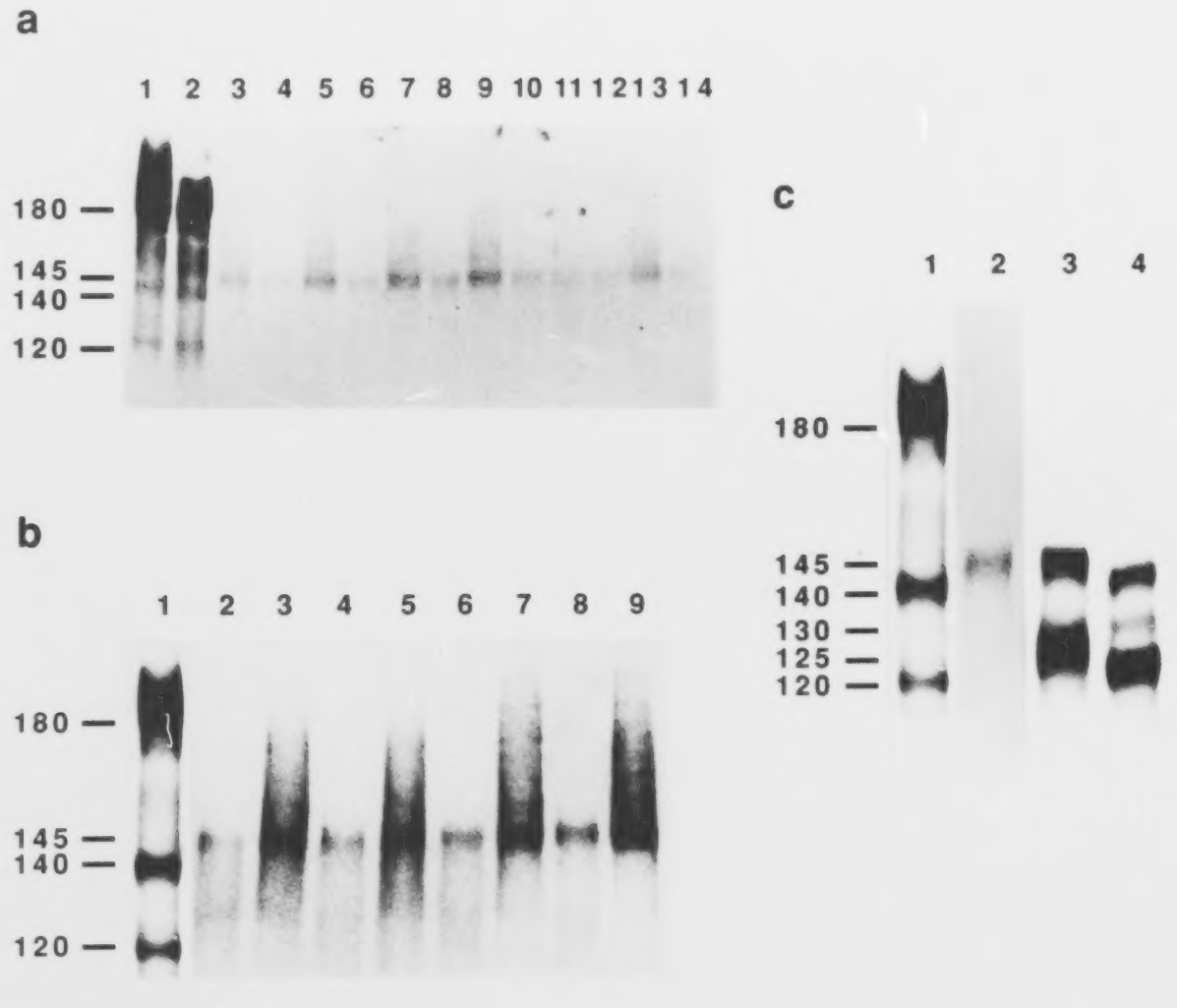

N-CAM
PSA
ACTIN
A
D
G
B
E
H
C
F

at the base of the filaments (Fig. II-3F) to being on smooth muscle cells in the dermis (as in Fig. II-3I) (data not shown). PSA was not detected in the feather filaments at any stage.

Immunoblot analysis of N-CAM and PSA in developing feathers

To determine which molecular forms of N-CAM were present in developing feathers, we used Western blot analysis to compare immunoaffinity purified skin N-CAM, and N-CAM from crude homogenates of skin and filaments alone, to N-CAM isolated from embryonic brain (Fig. II-4a). Brain N-CAM from stage 41 (E15) embryos showed a broad band of high molecular weight (180-250 kD), indicating the presence of sialylated N-CAM (Fig. II-4a, lane 1). Removal of the PSA revealed three bands of 180, 140, and 120 kD (Fig. II-4a, lane 2). Immunoaffinity purified N-CAM from stage 41 (E15) skin showed a broad band extending upwards from 145 kD (Fig. II-4a, lane 3), which resolved into a single band of M_r=145 kD upon removal of PSA (Fig. II-4a, lane 4, compared to lane 2). The same 145 kD polypeptide was detected throughout feather development (Fig. II-4a, lanes 5 to 14). Since filaments do not contain any muscle tissue (see above), the 145 kD band seen in developing filaments could not be solely due to contamination of the homogenates with muscle that expresses a previously reported variant of N-CAM (Prediger et al., 1988). PSA was not detected in the feather filaments (Fig II-4a, lanes 11 and 12). No N-CAM could be detected in stage 44 (E18) feather filaments, which are essentially acellular (Lucas and Stettenheim, 1972) (data not shown). The small amounts

Figure II-4. Western blot of N-CAM from (a) skin at different stages of development, (b) separated epidermis and dermis and, (c) brain, skin, heart and skeletal muscle. (a) Affinity-purified N-CAM from stage 41 (E15) skin appears as a broad band extending upwards from 145 kD (lane 3) which, upon removal of PSA, resolves into single band of M_r=145 kD (lane 4) compared to the 180, 140, and 120 kD polypeptides in brain (lane 1, without boiling; lane 2, with boiling). The same M_r=145 kD polypeptide is seen throughout feather development (all paired lanes represent N-CAM without and with boiling, respectively) (E7 skin, lanes 5-6; E10 skin, lanes 7-8; E14 skin, lanes 9-10; E14 filaments alone, lanes 11-12; E18 skin, lanes 13-14). Comparison of N-CAM in E14 skin, without (lane 9) and with boiling (lane 10), to N-CAM in E14 filaments, without (lane 11) and with boiling (lane 12), shows that filament N-CAM does not contain PSA. (b) The epidermis also contains the M_r=145 kD polypeptide, and epidermal N-CAM does not contain PSA, unlike N-CAM from the corresponding dermis (lane 1 is E15 brain N-CAM; lanes 2, 4, 6, and 8 are E6-E9 epidermal N-CAM, respectively; lanes 3, 5, 7, and 9 are E6-E9 dermal N-CAM). (c) A similar-sized M_r=145 kD N-CAM polypeptide is seen in skin, heart, and skeletal muscle (lanes 2, 3, and 4) compared to the 140 kD polypeptide in brain (lane 1) (all samples were boiled). Anti-N-CAM 802 polyclonal antibody was the primary antibody for all blots shown. Marks on the left-hand side of the blots indicate the size of the protein bands in kD.


a
1 2 3 4 5 6 7 8 9 10 11 12 13 14
180
145
140
120
b
1 2 3 4 5 6 7 8 9
180
145
140
120
c
1 2 3 4
180
145
140
130
125
120

of 180 kD N-CAM polypeptide seen in some of the skin extracts is due to nerves present within the dermis; the 180 kD form of N-CAM is known to be restricted to nerves in developing feathers (Prieto et al., 1989).

To verify that the difference between the 140 kD N-CAM polypeptide in brain and the 145 kD N-CAM polypeptide in developing feathers was not due to a gel artifact, we ran multiple parallel lanes of immunoaffinity purified N-CAM from brain and skin on a gel and immunoblotted with a polyclonal anti-N-CAM antibody. We consistently found a N-CAM polypeptide in skin that was 145 kD compared to the 140 kD polypeptide in embryonic brain (data not shown).

In order to demonstrate the presence of the 145 kD N-CAM polypeptide and the absence of PSA in the epidermis, we performed Western blot analysis on extracts made from stage 29-35 (E6-E9) epidermis and compared them to those from the corresponding dermis. As Fig. II-4b shows, a single 145 kD N-CAM polypeptide was present in the epidermis from stage 29 (E6) to stage 35 (E9) (lanes 2, 4, 6, and 8), and did not contain any PSA, unlike N-CAM in the corresponding dermis (lanes 3, 5, 7, and 9).

The presence of a M_r=145 kD N-CAM polypeptide in developing feathers raised the question of whether this polypeptide was the same size as a M_r=145-150 kD polypeptide reported in other non-neuronal tissues such as embryonic heart and skeletal muscle (Murray et al., 1986; Moore et al., 1987). Comparison of the N-CAM polypeptides present in embryonic chick brain, feather filaments, heart, and skeletal muscle (Fig. II-4c) showed that all three non-neuronal tissues expressed a similar-sized M_r=145 kD polypeptide

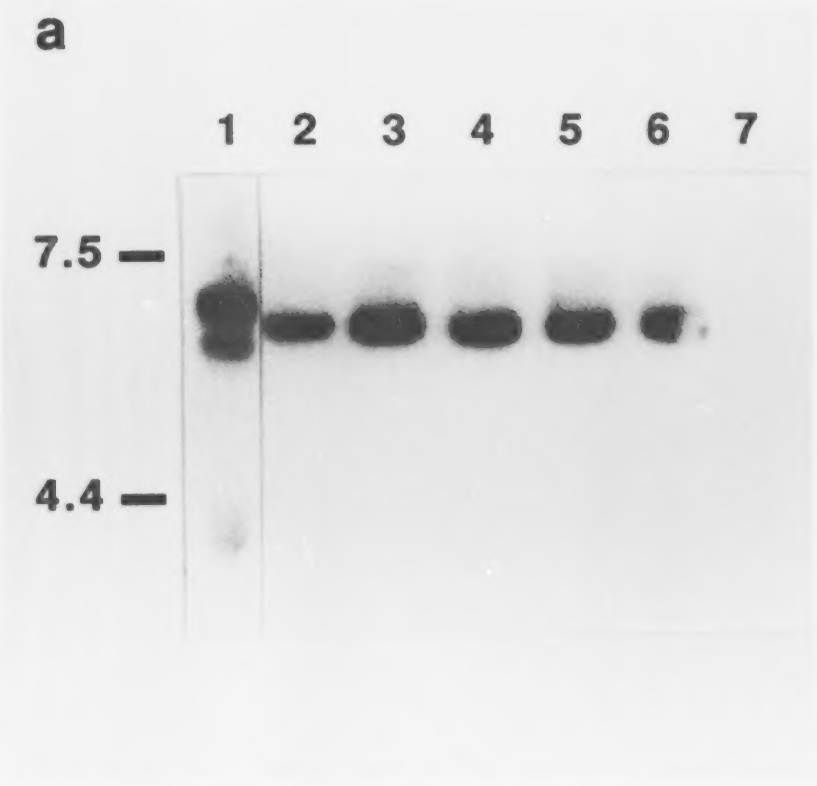
(Fig. II-4c, lanes 2-4) compared to the 140 kD polypeptide in brain (lane 1). Heart and skeletal muscle also expressed several smaller polypeptides of M_r=130 and 120-125 kD, as previously reported (Murray et al., 1986; Moore et al., 1987) (Fig. II-4c, lanes 3 and 4).

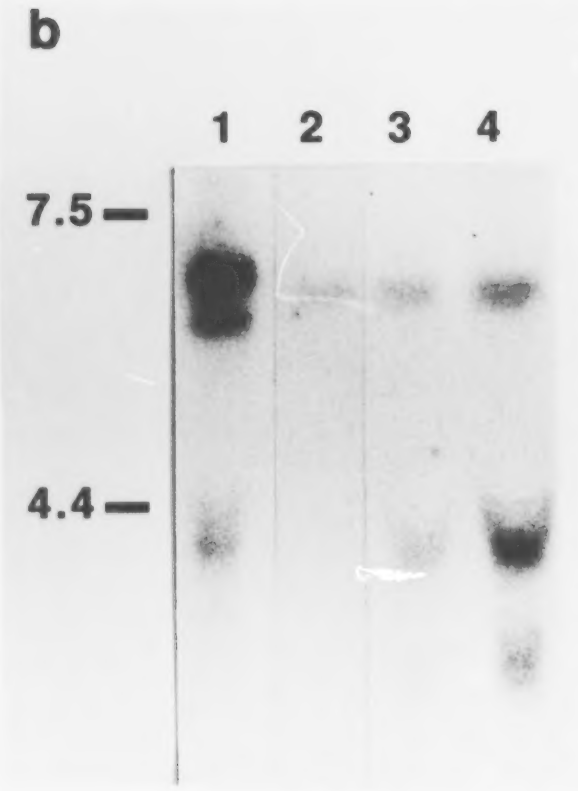
Northern blot analysis of N-CAM mRNA in developing feathers

We compared the N-CAM mRNA species present in feathers with those present in embryonic brain to determine if the M_r=145 kD polypeptide in feathers was encoded by a larger mRNA than the 140 kD polypeptide in brain. Throughout feather development, we detected a single mRNA species of 6.4 kb (Fig. II-5a, lanes 2 to 6), compared to 6.9, 6.1, and 4.0 kb mRNAs in embryonic brain (Fig. II-5a, lane 1). Embryonic liver contains little N-CAM after stage 30 (E7) and served as a negative control (Fig. II-5a, lane 7).

The presence of a 6.4 kb N-CAM mRNA species in developing feathers raised the question of whether a similar-sized transcript was present in heart and skeletal muscle, which also expressed a M_r=145 kD N-CAM polypeptide. Comparison of N-CAM mRNAs present in stage 41 (E15) feather filament, heart, and skeletal muscle (Fig. II-5b, lanes 2-4) showed that all three non-neuronal tissues contain a 6.4 kb mRNA, compared to the 6.1 kb mRNA in stage 41 (E15) brain (Fig. II-5b, lane 1).

Figure II-5. Northern blots (a) of N-CAM mRNA expressed during feather development (lane 1, E15 brain; lane 2, E7 skin; lane 3, E10 skin; lanes 4-5, E14 skin and filament alone; lane 6, E18 skin; lane 7, E15 liver), and (b) comparing N-CAM mRNA in E15 brain (lane 1), filament (lane 2), heart (lane 3), and skeletal muscle (lane 4). (a) A single 6.4 kb mRNA is present during feather formation and, (b) a similar-sized 6.4 kb mRNA is seen in all three non-neuronal tissues. In each case, 5 μg total RNA was hybridized to a DNA probe prepared from the 1210-bp Pst 1 fragment of pBS208. Size markers (in kb) are at the sides of the blots.


a
1 2 3 4 5 6 7
7.5
4.4


b
1 2 3 4
7.5
4.4

PCR analysis of exon splicing pattern and polyadenylation sites in feather N-CAM mRNA

In addition to the alternative splicing which generates the major neuronal N-CAM polypeptides (Owens et al., 1987) (see Fig. II-6A), splicing that alters shorter segments of the N-CAM polypeptide chains also occurs between exons 7-8 and 12-13 (Dickson et al., 1987; Gower et al., 1988; Prediger et al., 1988; Small et al., 1988; Santoni et al., 1989; Thompson et al., 1989; Small and Akeson, 1990; Reyes et al., 1991; Zorn and Krieg, 1992). In embryonic chicken, the only known alternative splice is a 93-bp insert, composed of four exons, that is present between exons 12-13 in heart and skeletal muscle N-CAM mRNA. This insert was detected by screening an embryonic heart cDNA library (Prediger et al., 1988).

The larger size of the 6.4 kb N-CAM mRNA species in feathers compared to the 6.1 kb mRNA in brain suggested that alternatively spliced exons might be present within the feather N-CAM mRNA. To determine whether or not the 93-bp alternative splice, or novel splices, were present in N-CAM of developing feathers and accounted for the larger size of the 6.4 kb mRNA, we used the polymerase chain reaction (PCR) to amplify and compare selected regions of N-CAM mRNA in embryonic chicken brain, filaments, heart, and skeletal muscle.

In order to find out where in the 6.4 kb N-CAM mRNA any alternative splices were present, we compared the PCR products generated by amplifying the regions between exons 1-8, 6-8, 6-13, 11-13, and 11-19 (see Fig. II-6A). The sizes of the products generated from brain RNA using our primers are 1086-bp for exons 1-8,

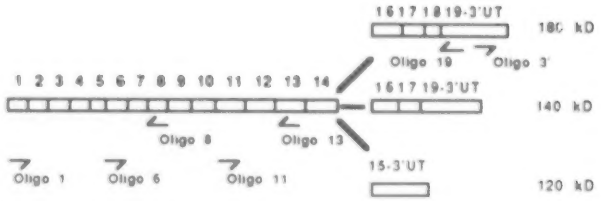
272-bp for exons 6-8, 1004-bp for exons 6-13, 270-bp for exons 11-13, and 871-bp (for 140kD N-CAM) or 1654-bp (for 180kD N-CAM) (Owens et al., 1987) for exons 11-19.

Comparison of the products from exons 1-8 (Fig. II-6C, lanes 1-4) and exons 6-8 (Fig. II-6D, lanes 1-4) showed that all four tissues lack alternative splices between exons 1-8; single bands of 1086-bp and 272-bp were detected in each tissue for the regions between exons 1-8 and 6-8, respectively. Restriction digests of the exons 1-8 and 6-8 PCR products confirmed that there were no differences in the products obtained from the four tissues (data not shown). Thus, unlike the alternative splicing of the 30-bp VASE (or π) insert at the exon 7-8 junction in rat brain and heart (Small et al., 1988; Small and Akeson, 1990; Reyes et al., 1991) and in many frog tissues (Zorn and Krieg, 1992), we find no evidence for alternative splicing in this region of chicken N-CAM, in agreement with previous investigations (Small et al., 1988). If VASE or other inserts are present in this region of chicken N-CAM, they must either be present in the N-CAM mRNA at levels below the sensitivity of our methods or be expressed at specific developmental stages that were not examined.

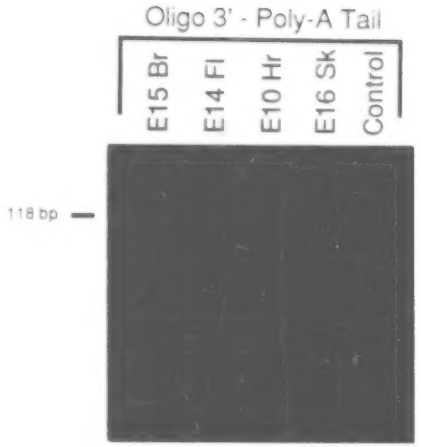
Amplification of the region between exons 6-13, 11-13, and 11-19 revealed an alternative splice in the non-neuronal tissues and the absence of this splice in brain. In particular, in all three non-neuronal tissues: (a) two bands of 1004-bp and 1097-bp were generated by amplifying the region between exons 6-13 (Fig. II-6C, lanes 6-8); (b) two bands of 270-bp and 362-bp were generated for the region between exons 11-13 (Fig. II-6D, lanes 6-8) and; (c) two bands of 871-bp and 964-bp were generated for the region between

Figure II-6. Analysis of exon splicing pattern and polyadenylation sites in skin N-CAM mRNA by using the polymerase chain reaction (PCR). (A) Schematic illustration of primers used for PCR reactions, and their relation to the mRNAs encoding the 180, 140, and 120 kD brain N-CAM polypeptides. Numbers above open boxes represent exons 1-19 as indicated. Exons 1-14 are shared by all N-CAM mRNAs. Exon 15 is present only in the 120 kD form, and exon 18 is present only in 180 kD form, as shown at the right. 3'-UT represents the 3' untranslated region up to and including the poly-A tail. (B) RACE PCR amplification from oligo 3' to the poly-A tail of E15 brain (Br), E14 filament (Fl), E10 heart (Hr), and E16 skeletal muscle (Sk) mRNAs. Controls with no template added did not generate any bands. (C) PCR amplification of the regions between exons 1-8, exons 6-13, and exons 11-19 in brain (Br), filament (Fl), heart (Hr), and skeletal muscle (Sk) mRNAs. (D) PCR amplification of the regions between exons 6-8 and exons 11-13 in brain (Br), filament (Fl), heart (Hr), and skeletal muscle (Sk) mRNAs. Controls with no template added did not generate any bands. For each gel, the sizes of the PCR products (in bp) are indicated at the left.

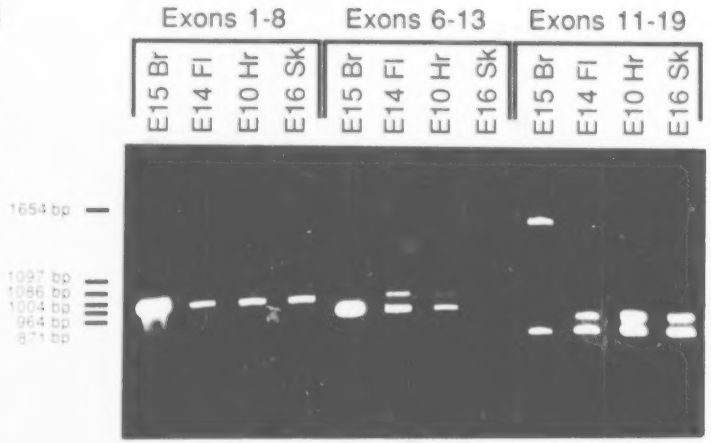
A


Schematic of Primers Used for PCR
16 17 18 19-3'UT
180 kD
Oligo 19
Oligo 3'
1 2 3 4 5 6 7 8 9 10 11 12 13 14
16 17 19-3'UT
140 kD
Oligo 8
Oligo 13
Oligo 1
Oligo 6
Oligo 11
15-3'UT
120 kD

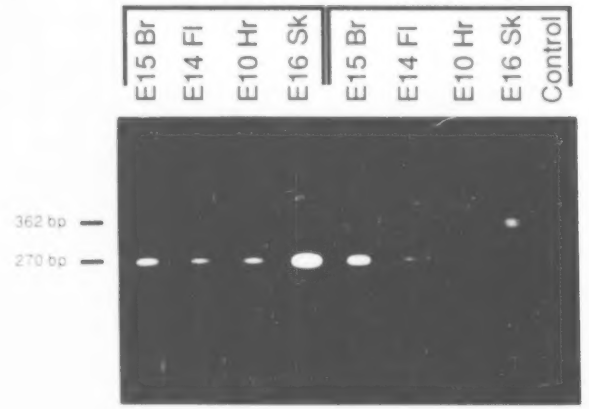
B


Oligo 3' - Poly-A Tail
E15 Br
E14 Fl
E10 Hr
E16 Sk
Control
118 bp

C


Exons 1-8
Exons 6-13
Exons 11-19
E15 Br
E14 Fl
E10 Hr
E16 Sk
1654 bp
1097 bp
1086 bp
1004 bp
964 bp
871 bp

D


Exons 6-8
Exons11-13
E15 Br
E14 Fl
E10 Hr
E16 Sk
Control
362 bp
270 bp

exons 11-19 (Fig. II-6C, lanes 10-12). The two bands of 871-bp and 1654-bp seen in Fig. II-6C, lane 9 (brain, exons 11-19) represent the mRNAs with and without exon 18, the splice that generates the 180 kD N-CAM polypeptide in brain (Owens et al., 1987) (see Fig. II-6A). Fig. II-6D, lane 9, represents a typical control in which no template was added to the PCR reaction. Restriction digests of the 6-13, 11-13, and 11-19 PCR products showed that a 93-bp insert was present between exons 12-13 (data not shown). We did not find evidence for differential expression of the four exons comprising the 93-bp insert, unlike the four exons comprising the 108-bp MSD1 insert in rat heart (Reyes et al., 1991) and the two exons comprising the 18-bp MSD insert in frog (Zorn and Krieg, 1992).

The 93-bp insert could not account for the observed difference in size of the 6.4 kb mRNAs in the non-neuronal tissues compared to the 6.1 kb mRNA in brain because two transcripts, with and without the insert, were present in the non-neuronal tissues based on PCR, yet only a single 6.4 kb band was seen on Northerns. We therefore looked for alternative polyadenylation sites in the 6.4 kb mRNAs that would increase the length of their 3' untranslated region. Rapid amplification of cDNA ends (RACE) PCR (Frohman et al., 1988) was used to selectively amplify the region between Oligo 3' (see Fig. II-6A) and the poly-A tail of the mRNAs (Fig. II-6B). A single 118-bp product was generated in all four tissues by amplifying this region (Fig. II-6B, lanes 1-4), indicating the same polyadenylation site was used in the 6.4 kb non-neuronal and 6.1 and 6.9 kb neuronal mRNAs. Fig. II-6B, lane 5, is a control with no template added to the PCR

reaction. Differential polyadenylation therefore does not account for the observed larger size of the 6.4 kb non-neuronal mRNAs compared to the 6.1 kb mRNA in brain.

Analysis of oligosaccharides attached to skin N-CAM

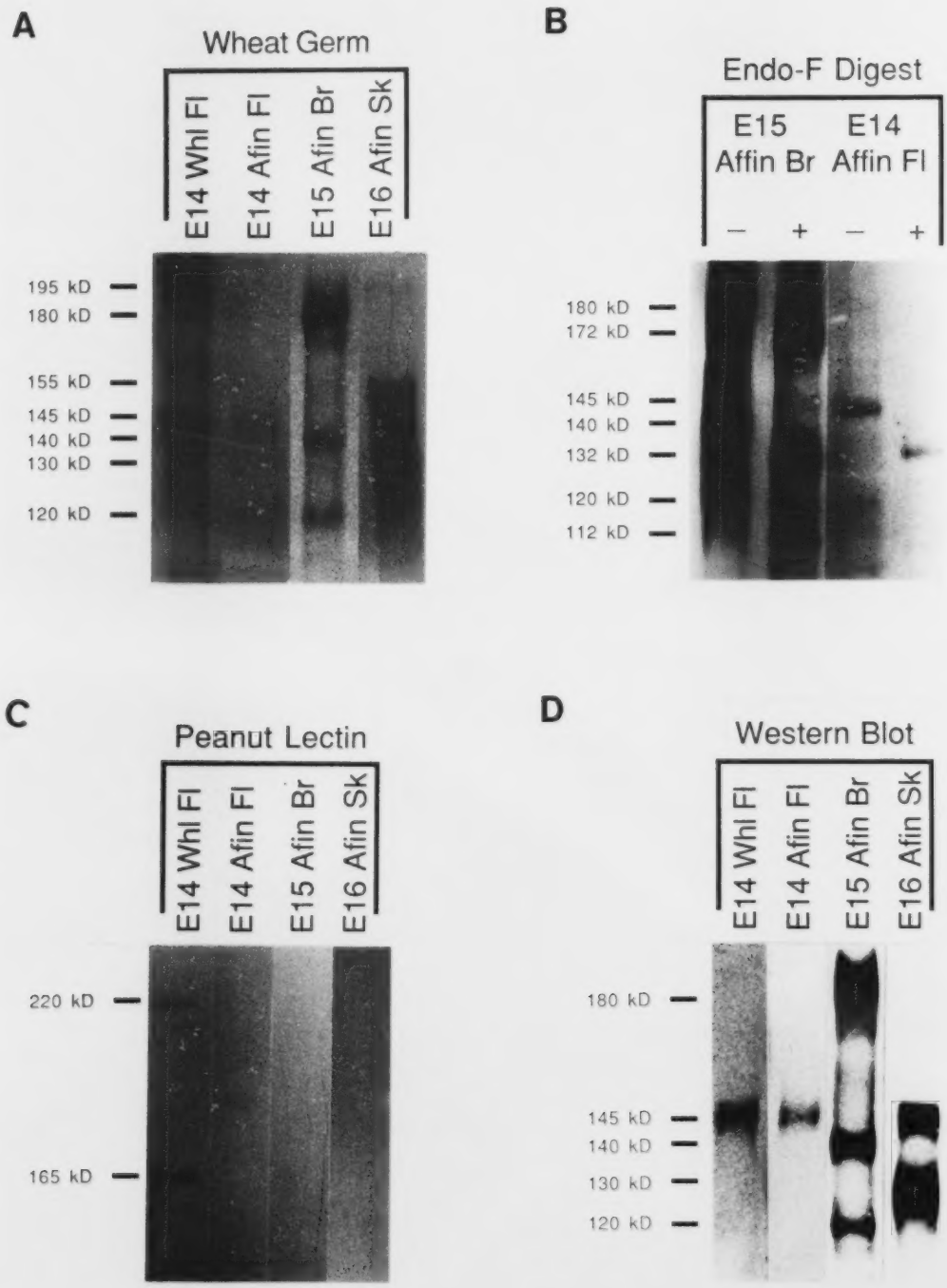
The identification of two 6.4 kb skin N-CAM mRNAs, with and without the 93-bp insert, means that two polypeptides, differing in size by about 3.4 kD, are probably present on immunoblots of skin N-CAM. As Fig. II-4a shows, these two polypeptides are not resolved by our gel system. Thus, the presence versus absence of the 93-bp insert can not account for the observed 5 kD size difference between the skin and brain polypeptides.

An alternative explanation for the increased size of the skin polypeptides is the attachment of carbohydrate to the proteins. N-CAM in chick brain has several sites for N-linked carbohydrate attachment, three of which are known to be glycosylated (Crossin et al., 1984; Cunningham et al., 1987). O-linked carbohydrate attachment to N-CAM has only been shown to occur on the 108-bp alternative splice MSD1 in human skeletal muscle N-CAM (Walsh et al., 1989). The 93-bp insert present in chicken skin is similar in structure to MSD1 (Prediger et al., 1988) and thus may serve as a site for O-linked attachment. However, since the 93-bp insert does not account for the size difference between the skin and brain polypeptides, O-linked carbohydrate that might be associated with this insert can also not be the basis of the size difference. We therefore first determined whether N-linked carbohydrate was present on the

skin N-CAM proteins by using lectin blot analysis with wheat germ agglutinin (WGA), which recognizes sugar linkages only in N-linked carbohydrate chains (Allen et al., 1973; Naquta and Berger, 1974).

WGA lectin blot analysis showed that N-linked carbohydrate was indeed present on the skin N-CAM polypeptides (Fig. II-7A). WGA bound to many N-linked proteins in whole filament extract, as expected (lane 1). However, in affinity-purified filament N-CAM, WGA bound to a single band corresponding to the 145 kD skin proteins (Fig. II-7A, lane 2; see also Fig. II-7D, lanes 1 and 2). Affinity-purified N-CAM from brain and skeletal muscle were blotted with WGA for controls because the 180, 140, and 120 kD brain N-CAM proteins, and the 145 and 130 kD muscle N-CAM proteins (see Fig. II-7D, lanes 3 and 4) are known to be N-glycosylated (Cunningham et al., 1983; Crossin et al., 1984; Barton et al., 1988). All three brain proteins were recognized by WGA as expected (Fig. II-7A, lane 3). Interestingly, in the skeletal muscle N-CAM, WGA bound to the 145 and 130 kD N-CAM proteins, but also bound to two other proteins of 155 and 190 kD (lane 4). These latter two proteins are not recognized by antibodies to N-CAM on Western blots (Fig. II-7D, lane 4). This suggests that other N-glycosylated proteins may interact with muscle N-CAM and remain attached to it during affinity purification. In support of this idea, it was recently found that N-CAM co-localizes with another cell adhesion molecule, N-cadherin, in avian skeletal myoblasts (Soler and Knudsen, 1991). Although neither the 155 kD nor the 190 kD band detected by WGA matches the known size of N-cadherin, it is possible that these two

Figure II-7. Analysis of carbohydrates attached to the skin N-CAM proteins by using lectins that specifically recognize N-linked (wheat germ agglutinin) or O-linked (peanut lectin) carbohydrate, and by selectively removing N-linked carbohydrate using endoglycosidase-F (endo-F). (A) Lectin blot analysis of whole-tissue extract (Whl) and affinity-purified (Afin) N-CAM from E14 filament (Fl), E15 Afin brain (Br) N-CAM, and E16 Afin skeletal muscle (Sk) N-CAM using wheat germ agglutinin. Samples pre-treated with endo-F did not show any staining (not shown). (B) Western blot showing changes in molecular weight of E15 Br and E14 Fl N-CAM polypeptides upon removal of N-linked carbohydrate by endo-F digestion (- and + are the undigested and digested samples respectively). (C) Lectin blot analysis of the same samples as in (A) but using peanut lectin. Staining with a different lectin that also recognizes O-linked carbohydrate (jacalin) gave the same result (not shown). Pre-treatment with endo-F did not affect the staining of either peanut lectin or jacalin (not shown). (D) Western blot analysis of the same samples as in A and C using anti-N-CAM 802 polyclonal antibody to show the N-CAM proteins present in each sample. For each of the blots, the sizes of the bands (in kD) are indicated at the left.


A
Wheat Germ
E14 Whl Fl
E14 Afin Fl
E15 Afin Br
E16 Afin Sk
195 kD
180 kD
155 kD
145 kD
140 kD
130 kD
120 kD
B
Endo-F Digest
E15 Affin Br
E14 Affin Fl
–
+
–
+
180 kD
172 kD
145 kD
140 kD
132 kD
120 kD
112 kD
C
Peanut Lectin
E14 Whl Fl
E14 Afin Fl
E15 Afin Br
E16 Afin Sk
220 kD
165 kD
D
Western Blot
E14 Whl Fl
E14 Afin Fl
E15 Afin Br
E16 Afin Sk
180 kD
145 kD
140 kD
130 kD
120 kD

proteins are associated with N-CAM in muscle and may play a role in regulating N-CAM-mediated adhesion. At present, the identity and function of these proteins remains unknown.

Knowing that N-linked carbohydrate was present on the skin proteins, the next step was to determine whether or not this carbohydrate actually accounted for the size difference between the 145 kD skin and 140 kD brain polypeptides. We therefore digested affinity-purified N-CAM from brain and filament with endoglycosidase-F (endo-F) (Elder and Alexander, 1982) to find out what changes in the size of the proteins occurred upon removal of the N-linked carbohydrate. Comparison of the brain and skin samples with and without endo-F digestion (Fig. II-7B) showed that the size difference between the 140 kD brain and 145 kD skin polypeptides could be accounted for by the N-linked carbohydrate attached to the skin proteins. A decrease in relative molecular weight of about 8 kD occurred upon digestion of the brain N-CAM polypeptides with endo-F (Fig. II-7B, lanes 1 and 2). In comparison, the skin proteins showed a decrease in relative molecular weight of about 13 kD upon endo-F treatment (compare lanes 3 and 4), and were a similar size to the 132 kD, endo-F-treated brain protein (compare lanes 2 and 4).

Although O-linked carbohydrate associated with the 93-bp insert can not account for the larger size of the skin polypeptides, O-linked carbohydrate could potentially be present at other sites on the skin N-CAM proteins and contribute to their larger size. To determine whether O-linked oligosaccharides were present on skin N-CAM, we did lectin blot analysis on affinity-purified filament N-CAM using

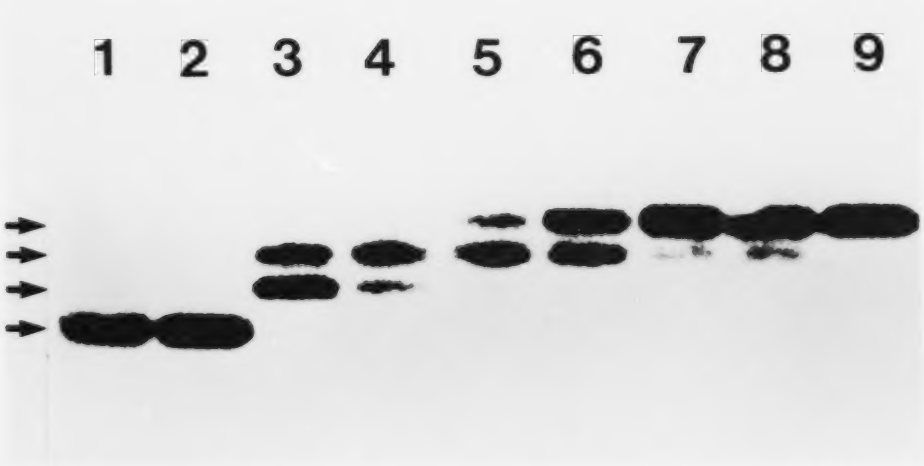
biotinylated peanut lectin (PNA), which recognizes sugar moieties found only in O-linked carbohydrate chains (Lotan et al., 1975) (Fig. II-7C). PNA bound to two proteins in whole-filament extract (Fig. II-7C, lane 1) but failed to bind to affinity-purified filament N-CAM (lane 2). PNA also failed to bind to affinity-purified N-CAM from brain and skeletal muscle (lanes 3 and 4). Jackfruit lectin (jacalin), which also binds to O-linked carbohydrate (Mahanta et al., 1990), gave identical results to PNA (data not shown). These results showed that skin N-CAM did not contain any O-linked carbohydrate and that the 5 kD larger size of the skin proteins was due exclusively to N-glycosylation. As well, these results suggested that the 93-bp insert in chicken muscle N-CAM was not O-glycosylated, unlike the MSD1 insert in human skeletal muscle, which serves as a site for O-linked attachment (Walsh et al., 1989). It is possible, however, that the 93-bp insert in chicken muscle may be O-glycosylated at other stages that were not examined.

To verify that the lectins we used were binding specifically to N-linked (WGA) and O-linked (PNA, jacalin) carbohydrates, we digested the whole-tissue extracts and affinity-purified N-CAM samples with endo-F prior to lectin blot analysis. Samples pre-treated with endo-F did not show any staining with WGA, indicating that the WGA was binding only to N-linked carbohydrate (data not shown). The endo-F treatment, however, did not affect the staining of the PNA or jacalin, indicating that these lectins were binding to O-linked carbohydrate (data not shown). Because the PNA and jacalin lectin used were biotinylated and were localized on blots using streptavadin, it was

possible that endogenous biotinylated proteins in skin might also be detected and give false bands. To ensure that non-specific binding of streptavadin was not occurring, parallel blots were incubated in streptavadin alone. No staining occurred on these blots, indicating that the bands present on the PNA and jacalin blots represented true proteins with O-linked carbohydrate (data not shown).

Because N-CAM in brain has several potential sites for N-linked carbohydrate attachment, only three of which are known to be glycosylated (Crossin et al., 1984; Cunningham et al., 1987), the question arose as to whether the larger size of the skin proteins was due to (a) an increased number of N-linked carbohydrate chains on these proteins compared to the brain proteins, i.e. extra sites were N-glycosylated on skin N-CAM, or (b) the same number of sites were used, but more oligosaccharide was present per chain. To differentiate between these two possibilities, we digested affinity-purified skin N-CAM with serial dilutions of endo-F to determine the number of N-linked chains present on the proteins (Elder and Alexander, 1982; Crossin et al., 1984). As Fig. II-8 shows, digestion of skin N-CAM with decreasing amounts of enzyme (lanes 2-8) generated a total of four discrete bands with increasing molecular weights (132 kD to 145 kD). The smallest and largest products were, respectively, completely digested skin N-CAM free from N-linked carbohydrate (lane 1) and skin N-CAM that was not digested (lane 9). Because the total number of species generated is always one greater than the number of N-linked carbohydrate attachment sites, the four bands generated by the serial digestion indicated that three N-linked attachment sites were present on the skin N-CAM proteins. The skin

Figure II-8. Determination of the number of N-linked carbohydrate chains present on the skin N-CAM proteins by using serial digestion with endoglycosidase-F (endo-F). Equal amounts of affinity-purified skin N-CAM were digested with decreasing amounts of endo-F. The samples were fractionated on a 4% polyacrylamide gel, transferred to nitrocellulose, and blotted with anti-N-CAM 802 polyclonal antibody. Skin N-CAM digested with 0.5 U endo-F for 48 hr was devoid of N-linked carbohydrate and had a molecular weight of about 132 kD (lane 1). Digestion with 1:4 serial dilutions of the amount of enzyme used in lane 1 for 48 hr generated four bands (lanes 2-8) with relative molecular weights of 132, 137, 141, and 145 kD (indicated by arrows). Undigested skin N-CAM (lane 9) had a molecular weight of 145 kD.


1 2 3 4 5 6 7 8 9

N-CAM proteins therefore contained the same number of N-linked chains as the brain N-CAM proteins (Crossin et al., 1984) and the larger size of the skin proteins was due to an increased amount of carbohydrate per chain, not to an increased number of individual chains.

Discussion

Cellular adhesion mediated by N-CAM is thought to be involved in a number of developmental processes, including neurite outgrowth, retinal histogenesis, nerve-muscle interaction, muscle development, kidney development, and formation of the feather (for reviews see Edelman et al., 1990). The role that cell adhesion plays in these processes is largely unknown, but changes in abundance, polypeptide structure, and glycosylation of N-CAM can modulate N-CAM-dependent cell-cell interaction and affect subsequent developmental events. Thus, determining precisely what forms of N-CAM are expressed during development is important in furthering our understanding of the role of N-CAM diversity in regulating cell adhesive interactions.

In the present study, we have investigated the forms of N-CAM that are expressed during feather formation as a first step towards examining the function of N-CAM, and the factors that may modulate its function, during the development of feathers. In particular, we have examined: (a) the distribution of N-CAM and PSA prior to and during feather formation, (b) the size of the N-CAM polypeptides expressed during feather morphogenesis and, (c) the exon splicing pattern of the mRNAs encoding the skin N-CAM proteins. Staining of

skin sections and wholemounts confirms previous descriptions of N-CAM distribution in developing feathers (Edelman and Chuong, 1985a), but reveals that N-CAM is expressed early on in the epidermis (as early as stage 29, E6) and that PSA is expressed only on a subset of the N-CAM in skin. Western and Northern blot analyses, in conjunction with PCR, show for the first time that two N-CAM polypeptides, both of M_r=145 kD, encoded for by two 6.4 kb mRNAs with and without a 93-bp insert between exons 12 and 13, are expressed and sialylated in a specific spatiotemporal manner in skin. Because two polypeptides are present in skin, yet only a single band is seen on Western blots, the 93-bp insert can not be responsible for the larger size of the skin proteins compared to the 140 kD N-CAM polypeptide in brain. We show that this size difference is instead due exclusively to an increased amount of N-linked carbohydrate attached to the skin N-CAM proteins. With regards to the larger size of the 6.4 kb skin mRNAs compared to the 6.1 kb mRNA in brain, the 93-bp insert can also not account for this size difference. We demonstrate that the same polyadenylation site is used in the 6.4 kb skin mRNA and in the 6.9 and 6.1 kb brain mRNAs, ruling out the possibility of a longer 3' untranslated region in the skin transcripts. On the basis of our results, we suggest that the larger size of the skin mRNAs may be due to use of a different transcriptional start site that is upstream of the start site used in brain N-CAM mRNA. Thus, we have shown that the simplest explanation for the increased skin N-CAM mRNA and protein size, namely the presence of an insert in the coding region, was not the cause of the observed differences.

Our observations demonstrate that the expression pattern of N-CAM changes from a uniform distribution in both epidermis and dermis to a localized expression in feather forming areas of the dorsal skin (epidermal placodes and dermal condensations). In the case of the epidermis, we show that N-CAM expression increases on cells forming the epidermal placode and decreases on non-feather forming cells in this tissue layer.

The presence of N-CAM in both the epidermis and dermis, as well as the coordinated restriction of N-CAM expression in both tissues to cells involved in feather formation, strengthens the idea that N-CAM-mediated adhesion is important for feather development. Feather formation depends upon a series of interactions between the epidermis and dermis to regulate the development and patterning of the feather structures (Sengel, 1958; Rawles, 1963; Dhouailly, 1973; Sawyer and Fallon, 1983). The coordinated expression of N-CAM in the placodes and condensations at stage 35 (E9) raises the possibility that N-CAM could be involved in contact and communication between the cells of the two tissue layers at this stage. Such direct contact and communication between the two tissue layers, although occurring long after interactions involved in patterning of the feather rudiments (Davidson, 1983a, b), could be involved in morphogenesis of individual feathers. In support of this idea is the requirement for direct contact between epidermis and dermis at this stage for normal filament formation (Konig and Sawyer, 1985). We are currently testing the above idea by perturbing N-CAM-mediated adhesion at different stages of feather development using specific antibodies.

We have used a mAb that specifically recognizes polysialic acid (PSA) to examine in detail the expression of PSA on N-CAM of developing feathers. We find that the PSA content of N-CAM does not start out high and decrease gradually as feather development proceeds (E to A conversion), but rather that PSA is present transiently on N-CAM in the dermal condensations (stage 35-36, E9-10) and is present for a long period on N-CAM of smooth muscle cells at the base of the feather filaments (stage 37-44, E11-18). The long-term expression of N-CAM and PSA on the smooth muscle cells in the dermis is a striking contrast to previous reports of N-CAM on smooth muscle of other organs, which suggest N-CAM is either not expressed at all (Edelman et al., 1983; Edelman, 1985) or is only transiently expressed on this cell type (Akeson et al., 1988).

The PSA component of N-CAM is thought to inhibit adhesion between cells and modulate other contact-dependent cellular events (Rothbard et al., 1982; Hoffman and Edelman, 1983; Acheson and Rutishauser, 1988; Rutishauser et al., 1988; Acheson et al., 1991). When the outgrowth of the filaments begins in developing feathers, cell division occurs primarily in the caudal region of the feather rudiment (Wessells, 1965; Desbien et al., 1991). Our results show that PSA is expressed primarily in the cephalic portion of the dermal condensations, which undergoes relatively little cell proliferation. In embryonic retina, the abnormal histogenesis caused by removal of PSA with endoneuraminidase-N (endo-N), an enzyme which cleaves α-2,8-polysialic acid (Vimr et al., 1984), is thought to be due to an increase in cell division (Rutishauser et al., 1985). This suggests that the restricted expression of PSA to the cephalic region of the feather

rudiment could be involved in the lower rate of cell division in this region. We are currently testing this hypothesis by culturing different stages of developing skin in the presence of endo-N.

Analysis of the N-CAM mRNA from skin demonstrates that the single 6.4 kb band seen on Northern blots actually consists of two species that differ by the presence versus absence of a 93-bp insert between exons 12 and 13. This small difference, which could not be resolved on the Northern blots, was shown by PCR analysis of skin N-CAM mRNA. Although the 93-bp insert is present in skin N-CAM, we showed that the size difference between the 6.4 kb skin and 6.1 kb brain mRNAs was not due to the presence of this, or other alternative splices in the coding region of the skin transcripts, nor to the use of different polyadenylation sites that could increase the 3' untranslated region of the skin mRNAs. These results, in conjunction with the findings of other investigators (Barthels et al., 1987; Hirsch et al., 1990), suggest that the use of differential transcriptional start sites may be the basis for most of the 300-bp size difference. At least two different start sites have been found in mouse brain N-CAM and appear to be used indiscriminately by different cell types during development (Barthels et al., 1987; Hirsch et al., 1990). Recent evidence suggests that different transcriptional start sites are also present in chicken N-CAM (Colewell et al., 1991; Brackenbury, 1992). Interestingly, approximately 330-bp upstream of the major start site used in chick brain N-CAM, there is a TATA-box that could serve as an alternative start site in other chick tissues (Brackenbury, 1992). The position of this TATA-box corresponds well with our observed 300-bp size difference between the skin and brain N-CAM

mRNAs. To date, we have been unsuccessful at determining whether a different start site is used in skin N-CAM due to the fact that primer extension at the 5' end of the skin mRNAs is confounded by the high GC content of this region. It will be necessary to use nuclease protection experiments to unambiguously establish the 5' end of the 6.4 kb skin N-CAM mRNAs and to verify the use of the upstream TATA-box or other start sites.

Just like for the N-CAM mRNAs, the inability to resolve the two skin N-CAM polypeptides on Western blots also means that the 93-bp insert does not account for the observed 5 kD difference between the 145 kD skin and 140 kD brain N-CAM proteins. An alternative explanation for the increased size of the skin polypeptides is the attachment of carbohydrates to the skin proteins. Several sites for N-linked carbohydrate attachment are known to be present in chick N-CAM (Crossin et al., 1984; Cunningham et al., 1987), but the only evidence for O-linked carbohydrate attachment is associated with the MSD1 insert in human skeletal muscle N-CAM (Walsh et al., 1989). MSD1 is almost identical to the chick 93-bp insert for approximately the second half of its sequence, suggesting that the 93-bp insert may also serve as a site for O-linked attachment. However, because the two skin proteins differ only by the presence versus absence of the 93-bp insert, and thus potentially differ by the presence or absence of O-linked carbohydrate attached to this insert, splice-specific O-linked sugars can not be the basis for the larger size of the skin polypeptides. Using lectins (PNA and jacalin) that specifically recognize sugar moieties present in O-linked carbohydrates, we also ruled out the possibility of O-linked oligosaccharides being present

at other locations on skin N-CAM and contributing to the size of the proteins. We therefore checked if N-linked carbohydrates were present on skin N-CAM using a lectin (WGA) that recognizes N-linked sugar moieties, and we also determined what changes in the size of the skin polypeptides occurred upon removal of N-linked carbohydrates using endo-F (Elder and Alexander, 1982). Blotting with WGA showed that N-linked carbohydrate was indeed present on the skin N-CAM proteins, and digestion with endo-F showed that the 5 kD size difference between the skin and brain proteins could be accounted for by this N-linked carbohydrate. These findings corroborate our suggestion that the 93-bp insert does not account for the larger size of the skin N-CAM polypeptides.

We have shown that three N-linked carbohydrate chains are attached to skin N-CAM, which is the same number of chains present on brain N-CAM (Cunningham et al., 1983; Crossin et al., 1984). This suggests that the larger size of the skin proteins is due to the presence of extra carbohydrate per N-linked chain rather than the presence of additional N-linked chains at other sites on the skin polypeptides. The significance of the extra carbohydrate on the skin proteins is unknown. In frog olfactory neuroepithelium, many different N-linked forms of N-CAM have recently been discovered (Key and Akeson, 1991). Differential N-glycosylation of N-CAM may be involved in the regenerative plasticity of this system, perhaps by modulating N-CAM homophilic binding or functions of the individual N-CAM proteins (Key and Akeson, 1991). It is possible that N-CAM in skin may also be differentially N-glycosylated during feather formation, and that variation in the N-linked carbohydrate attached

to the skin proteins may regulate their function in this system. To examine the role of N-linked carbohydrate on skin N-CAM it will be necessary to determine where the chains are attached to the proteins, what the structure of the chains is, and what the mechanisms are that regulate N-glycosylation in developing feathers.

In summary, we have described a number of structural variations in N-CAM mRNA and protein that may be important in modulating the function of N-CAM in developing feathers. These include: (a) splice variants that give rise to two different polypeptide chains, (b) variation in the amount of N-linked carbohydrate attached to the skin N-CAM proteins, (c) variation in the amount of PSA attached to the skin N-CAM proteins and, (d) possible use of an alternative transcriptional start site.

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CHAPTER III

Perturbation of N-CAM and PSA in Developing Feathers

Introduction

Embryonic development proceeds under the driving force of the five primary cellular processes of adhesion, division, migration, death, and differentiation. During embryogenesis, morphogenetic movements bring cell populations together in a continuous set of interactions that involve cell contact and other molecular signals (Saxen et al., 1980). The interaction of these cell populations at specific times and locations leads to changes in gene expression, which in turn leads to cellular differentiation and eventually to the establishment of form. Clearly, there must be mechanisms that allow cells to specifically recognize and interact with one another, and there must also be developmental regulation of these mechanisms so that the proper series of interactions can occur. In addition, because adhesion, division, migration, death, and differentiation work together to establish form, there should be ways in which these processes are interconnected so that they can be coordinated during morphogenesis.

Of the five primary cellular processes, adhesion is thought to play a central role in development by mediating interactions between cells and by regulating the other cellular processes. This idea, called the regulator hypothesis (Edelman, 1984a), suggests that genes for cell adhesion molecules (CAMs) are expressed prior to the genes involved in differentiation, allowing CAMs to direct movement of cell populations within the embryo and determine which populations

interact. CAMs regulate cellular interaction by mediating direct contact between the plasma membranes of apposed cells, allowing for exchange of signals through gap junctions and through interaction of cell-surface ligands and receptors on different cells (Edelman, 1983, 1986; Acheson and Rutishauser, 1988; Keane et al., 1988). By varying the expression pattern of CAMs or their adhesive properties, or both, during morphogenesis, contact-dependent cellular events can be regulated, perhaps leading to changes in the other primary cellular processes and allowing cells of different histories to be brought together.

One of the best characterized CAMs in the developing embryo is the neural cell adhesion molecule (N-CAM). N-CAM is a family of proteins that are generated from a single gene by alternative splicing of mRNA (Murray et al., 1986). The N-CAM proteins are expressed in specific spatiotemporal patterns in many tissues during embryogenesis, these patterns often being coincident with inductive events between cell populations (Thiery et al., 1982; Edelman et al., 1983; Crossin et al., 1985; Chuong and Edelman, 1985a, b). Each of the N-CAM proteins has a distinct expression pattern (Pollerberg et al., 1985; Daniloff et al., 1986; Moore et al., 1987), suggesting that there may be differences in function between the polypeptides. These functions may be modified by the attachment of a carbohydrate moiety, polysialic acid (PSA), which decreases N-CAM-mediated adhesion and other contact-dependent interactions by preventing membrane contact between apposed cells (Rothbard et al., 1982; Hoffman and Edelman, 1983; Rutishauser et al., 1988; Acheson et al., 1991). At present, the functions of the individual N-CAM proteins

are not well understood. It is therefore of interest to determine which N-CAM proteins are restricted to particular tissues during development, and to determine the effects of perturbing the adhesion mediated by these proteins in these tissues. By correlating changes in normal morphogenetic events with the perturbation of specific N-CAM forms, it should be possible to gain insight into the functions of the individual N-CAM proteins.

The function of N-CAM in the formation and patterning of cell populations within a tissue can be examined during feather formation in embryonic chicken. During feather development, cells in the dermis proliferate and condense into a defined pattern of cell groups called dermal condensations (Davidson, 1983a). Each condensation is overlaid by a thickening in the epidermis called a placode, which together with the condensation constitutes an individual feather rudiment (Davidson, 1983a; Mayerson and Fallon, 1985). Within each rudiment, the dermal and epidermal cells interact and develop into a feather bud, which then elongates due to cell division and forms a feather filament (Wessells, 1965; Sengel, 1976; Sawyer and Fallon, 1983). Cells within the filament rearrange into a series of ridges that are parallel to its long axis (Lucas and Stettenheim, 1972). These ridges will develop into the branches, called barbs and barbules, of the mature feather.

During feather morphogenesis, N-CAM is expressed on cells involved in formation of the feather rudiments and filaments (Chuong and Edelman, 1985a; Marsh and Gallin, 1992). Specifically, N-CAM is expressed on cells that form the epidermal placodes and dermal condensations, on epidermal cells from which the filament

arises, and on epidermal cells thought to die in the filaments to generate spaces between the barbs and barbules. Thus, N-CAM is present on cells that are dividing, and perhaps even on cells undergoing programmed death, suggesting that N-CAM-mediated adhesion may regulate these events and may be involved in directing feather morphogenesis.

As a first step towards determining the role of N-CAM-mediated adhesion in feather formation, we analyzed the structure of N-CAM in the feather (Marsh and Gallin, 1992). Our results indicated that two 145 kD N-CAM proteins, with and without a 31 amino acid insert in the extracellular domain, were expressed throughout feather development. As well, we found that PSA was expressed on only a subset of the cells that expressed N-CAM, in particular on cells in the dermal condensations (stage 35-36, E9-E10) and on smooth muscle cells in the dermis (stage 37-44, E11-E18). Our findings suggested that N-CAM might be involved in epidermal-dermal interaction and in formation and patterning of the feather rudiments and filaments, and that PSA might regulate cell division in the feather bud at the time of filament outgrowth (Marsh and Gallin, 1992).

The effect of perturbing N-CAM-mediated adhesion has been examined in several developing tissues and in cultures of various cell lines by using antibodies that inhibit N-CAM homophilic binding (Buskirk et al., 1980; Fraser et al., 1984; Keilhauer et al., 1985; Maier et al., 1986; Knudsen et al., 1990; Aoki et al., 1991; Lanier et al., 1991; Jiang and Chuong, 1992) and endoneuraminidase-N (endo-N) to selectively remove PSA (Rutishauser et al., 1985; Landmesser et al., 1990). The results of these experiments support the regulator

hypothesis in that interfering with N-CAM-mediated adhesion and removing PSA causes changes in the primary cell processes in cultured cells (Aoki et al., 1991; Lanier et al., 1991) and alters the morphology and histology of treated tissues (Buskirk et al., 1980; Fraser et al., 1984; Rutishauser et al., 1985; Maier et al., 1986; Jiang and Chuong, 1992). Thus, in addition to mediating interaction between cells, N-CAM may also function in modulating and in coordinating the other primary cell processes during morphogenesis.

The effect of perturbing N-CAM-mediated adhesion in developing feathers was recently examined using antibodies against N-CAM (Jiang and Chuong, 1992). Organ cultures of skin treated with anti-N-CAM antibodies showed altered morphology of the feather rudiments (Jiang and Chuong, 1992), suggesting that N-CAM was involved in rudiment formation. The effect on feather development of removing PSA was not examined in this study. To further investigate the function of N-CAM-mediated adhesion in developing feathers, and to investigate the function of PSA, we treated organ cultures of skin with an antibody known to inhibit N-CAM homophilic adhesion, and with endo-N to remove PSA, and looked at the short-term and long-term effects of the treatments on morphology and histology of the feather rudiments and of the filaments that developed from these rudiments.

In contrast to the results of Jiang and Chuong (1992), we did not observe alterations in the feather rudiments upon treatment with our anti-N-CAM antibody. In fact, neither our antibody nor our endo-N treatment caused any apparent change in the feather rudiments or feather filaments. We suggest that the lack of effect

of our antibody treatment compared to the antibody treatment of Jiang and Chuong (1992) may be due to differences in the N-CAM epitopes bound by the antibodies used in the two studies, and that the lack of effect of our endo-N treatment may be due to PSA being involved in feather erector muscle development within the dermis and not in feather morphogenesis directly.

Materials and Methods

Materials - Reagents were purchased from the sources indicated: materials for SDS-PAGE (Bio-Rad); NCTC 135, gentamycin sulfate, and goat and rabbit serum (Gibco); antibodies (Bio-Rad, ICN Immunobiologicals, or Jackson Immunoresearch); Tissue-Tek O.C.T. compound (Miles Scientific); 0.45 µm HA Filter (Millipore). All other reagents were obtained from BDH or Sigma.

Bouin's fixative and carmine borate stain were prepared as described by Watterson and Schoenwolf (1984). Tris buffered saline (TBS), TBS with Tween 20 (TTBS), chromogen diluent, and phosphate buffered saline (PBS) were prepared as described previously (Marsh and Gallin, 1992).

Fertile White Leghorn eggs were obtained from the University of Alberta Experimental Farm, Poultry Division. Eggs were incubated at 37°C, 55% relative humidity, for up to 18 days. Embryos were staged according to the method of Hamburger and Hamilton (1951). E7 to E16 denote embryonic age from 7-days to 16-days after the onset of incubation.

Antibodies - Anti-N-CAM 802 polyclonal antibody, anti-N-CAM CAM6 monoclonal antibody (mAb), anti-PSA 5A5 mAb, anti-L-CAM 6H1 mAb, and anti-α–actin αsm-1 mAb were used as described previously (Marsh and Gallin, 1992). In all cases, an irrelevant mAb against chick liver bile cannaliculi, culture supernatant from SP2/0 myeloma cells, and rabbit or goat serum were used as controls. For the vesicle aggregation assay, a rat anti-N-CAM polyclonal antibody (RaCC IgG) that inhibits N-CAM homophilic binding, and normal rat (pre-immune) IgG (NR IgG) were supplied by Dr. Ann Acheson, Department of Anatomy and Cell Biology, University of Alberta. Anti-PSA 5A5 mAb was obtained from the Developmental Studies Hybridoma Bank (Baltimore, MD).

Purification of Endoneuraminidase-N - Endo-N was purified using materials and a protocol supplied by Dr. Eric Vimr, Department of Veterinary Pathobiology, University of Illinois at Urbana-Champaign. The protocol used was a shortened version of the method of Hallenbeck et al. (1987). Briefly, *E. coli* EV36 were infected with K1F phage and the lysate was collected and precipitated with ammonium sulfate. Precipitate was collected by centrifugation and redissolved in 10 mM Tris, pH 7.6. Debris was pelleted by centrifugation, and the supernatant was collected and centrifuged at high speed to remove phage. Phage-free supernatant was dialyzed against 10 mM Tris, pH 7.6, and fractionated on a DEAE-Sephacel column in the same buffer, using a 0 to 0.4 M NaCl linear gradient. Every second fraction was assayed for endo-N activity as described previously (Hallenbeck et al., 1987). Positive fractions were pooled and precipitated, and the precipitate was collected by

centrifugation. The pellet was dissolved in 10 mM sodium phosphate buffer, pH 6.8, and dialyzed against the same buffer. Dialysate was incubated with hydroxyapatite overnight, and the supernatant was precipitated. Precipitate was collected by centrifugation, resuspended in 10 mM Tris, pH 7.6, and dialyzed against the same buffer. Dialysate (purified enzyme) was stored at -20°C in 50% glycerol. Yields were consistently 30000 U/ml.

Vesicle aggregation assay for CAM6 mAb inhibition of N-CAM homophilic binding - Brains dissected from stage 40 (E14) embryos were homogenized in PBS containing 100 KIU/ml aprotonin (PBS/A) (5 ml/brain) using a Dounce homogenizer, and centrifuged at 1000 x g for 5 min at 4°C to pellet nuclei and debris. Supernatant was centrifuged at 17000 x g for 20 min at 4°C, and the pellet was resuspended in 20 ml of PBS/A plus 0.6 M sucrose. The suspension was transferred to an ultracentrifuge tube over a 5 ml cushion of PBS/A plus 1.2 M sucrose. Tubes were spun in a Ti60 rotor at 35000 rpm for 45 min at 4°C, and the vesicle fraction was collected from the interface of the two sucrose concentrations. Vesicles were washed with 20 ml of PBS/A, centrifuged at 17000 x g for 20 min at 4°C, and resuspended in 10 x pellet volume of PBS/A containing 50 µg/ml DNase and 1 mM EDTA (PBS/A/D/E).

For the aggregation assay, the following samples were set up in microfuge tubes: (a) 40 µl of vesicle suspension alone, (b) 40 µl of vesicles plus 300 µg of normal rat IgG (NR IgG), (c) 40 µl of vesicles plus 300 µg of anti-N-CAM polyclonal (RaCC IgG), and (d) 40 µl of vesicles plus 300 µg of anti-N-CAM CAM6 mAb. PBS/A/D/E was added to each sample to bring the total volume to 200 µl, and all

samples were incubated on ice for 15 min. During this time, four pairs (duplicate sets) of scintillation vials containing 500 µl of PBS/A/D/E plus 500 µg/ml BSA were equilibrated to 37°C on a rotary shaker. After the 15 min incubation, 50 µl of each sample was added to a set of duplicate vials. Upon adding the sample, each vial was swirled and 50 µl was immediately removed and added to a microfuge tube containing 100 µl of 1% glutaraldehyde in PBS (zero time point). The scintillation vials were then incubated on the rotary shaker at 37°C, 70 rpm, and additional 50 µl samples were removed and fixed after 15 min and 30 min. To prevent disturbance of the aggregates, samples were collected using cut-off pipette tips.

To assess the extent of aggregation, vesicles were placed onto glass slides and examined using an inverted microscope. Aggregation was scored using a semi-quantitative system, '0' representing free vesicles and no aggregates, '0.5' free vesicles and only a few aggregates, '1' half vesicles and half aggregates, and '1.5' aggregates only. One sample from each duplicate set was scored first, and then the second sample was scored. Each set was scored by two observers. Using this system, a maximal score of 3 was possible. The sums from both observers were averaged, and the % inhibition of aggregation was calculated as follows: % inhibition = [1 - (average of sums/3)] x 100. Using this calculation, 0% inhibition occurred with vesicles alone, 10% inhibition occurred with NR IgG, 50% inhibition occurred with RaCC IgG, and 80% inhibition occurred with CAM6 mAb after 30 min.

Organ cultures and chorioallantoic membrane grafts - For 3-day and 8-day organ cultures, dorsal skin from stage 30 (E7) chicks was removed by blunt dissection, placed dermal side down onto a strip of HA filter that had been sterilized and soaked in NCTC 135 with 10 μg/ml gentamycin sulfate (medium), and spread such that no folding was evident. Skin and filter were placed onto a sterile, stainless steel grid in a plastic organ culture dish, and medium alone or medium containing various amounts of endo-N, boiled endo-N, CAM6 mAb, or endo-N plus CAM6 was added such that the skin explant was situated at the air-medium interface. Cultures were incubated at 37°C in a 10% CO_2 atmosphere for 3 or 8 days, with medium being changed daily. Enzyme and antibody were mixed into the medium just prior to addition to the organ cultures.

Because normal feather filament formation will not occur unless the skin becomes vascularized, untreated and treated 3-day organ cultures were grafted onto the chorioallantoic membrane (ChAM) of 10-day chick embryos and allowed to grow for an additional 4 or 8 days. Briefly, a hole was cut in the shell directly above the air space of the egg, the overlying shell membrane was removed with forceps, and the skin and filter were placed on top of a large blood vessel within the exposed ChAM. The hole was sealed with cellophane tape, and the egg was incubated at 37°C, 55% relative humidity, for 4 or 8 days. To ensure that the growing filaments did not adhere to the skin surface or to one another, ChAM grafts were moistened every 2 days with medium alone or with medium containing endo-N or CAM6 mAb as appropriate.

Gross morphology - Organ cultures and ChAM grafts were fixed in Bouin's fixative overnight, soaked in several changes of 70% ethanol saturated with lithium carbonate until no yellow color remained, stained in carmine borate overnight (Watterson and Schoenwolf, 1984), washed in acid alcohol until excess stain was removed, and stored in 70% ethanol. Samples were photographed on Kodak Tech Pan 100 film using a Wild dissecting microscope.

Histology - The same organ cultures and ChAM grafts photographed for gross morphology were dehydrated through a graded alcohol series, cleared in chloroform, embedded in Paraplast Plus (Fisher), and sectioned at 5 μm on a microtome. Sections were stained with hematoxylin and eosin (Luna, 1968) and photographed on Kodak Tech Pan 100 film using a Zeiss Axiophot microscope.

Immunoperoxidase and Immunofluorescent staining - Staining of wholemounts and sections was carried out as described previously (Marsh and Gallin, 1992). In the CAM6 mAb treated cultures and grafts, N-CAM was localized using rabbit anti-N-CAM 802 polyclonal antibody in order to avoid staining from the mouse CAM6 mAb present in the tissue.

Culture extracts, endo-N digestions, and immunoblots - Protein extracts of untreated and treated cultures and grafts were made as described previously (Marsh and Gallin, 1992). For removal of PSA, affinity-purified brain or filament N-CAM was incubated with 50 U endo-N in PBS, pH 7.2, for 1 h at 37°C in a total volume of 50 μl. Controls were run by replacing enzyme with PBS or with 50 U boiled endo-N. SDS-PAGE and immunoblotting were carried out as described previously (Marsh and Gallin, 1992).

Results

The terminology used in describing feather structures and the processes involved in feather formation is as outlined by Chuong and Edelman (1985a) and Lucas and Stettenheim (1972).

For the experiments described below, short-term and long-term effects of the different treatments (CAM6 mAb, endo-N, and both together) on feather rudiment development were examined by organ culturing stage 30 (E7) skin for 3 or 8 days respectively, and short-term and long-term effects on filament formation were examined by ChAM grafting 3-day organ cultures for 4 or 8 days respectively. In all cases, the organ cultures were incubated for the entire 3 or 8 day periods in medium containing mAb, endo-N, or both reagents as appropriate, and the ChAM grafts were likewise moistened over the entire 4 or 8 day periods with medium containing mAb, endo-N, or both reagents. The time periods chosen for the organ cultures and ChAM grafts were based on previous observations of the time required for rudiment formation and patterning to occur in vitro (Davidson, 1983a), and for filament formation to occur on ChAM grafts (Brotman, 1980).

Morphology and Histology of Normal Cultures and Grafts

In order to examine the short-term and long-term effects of perturbing N-CAM-mediated adhesion in developing feathers, it was first necessary to establish controls by examining the morphology and histology of feather rudiments and filaments from untreated organ cultures and ChAM grafts.

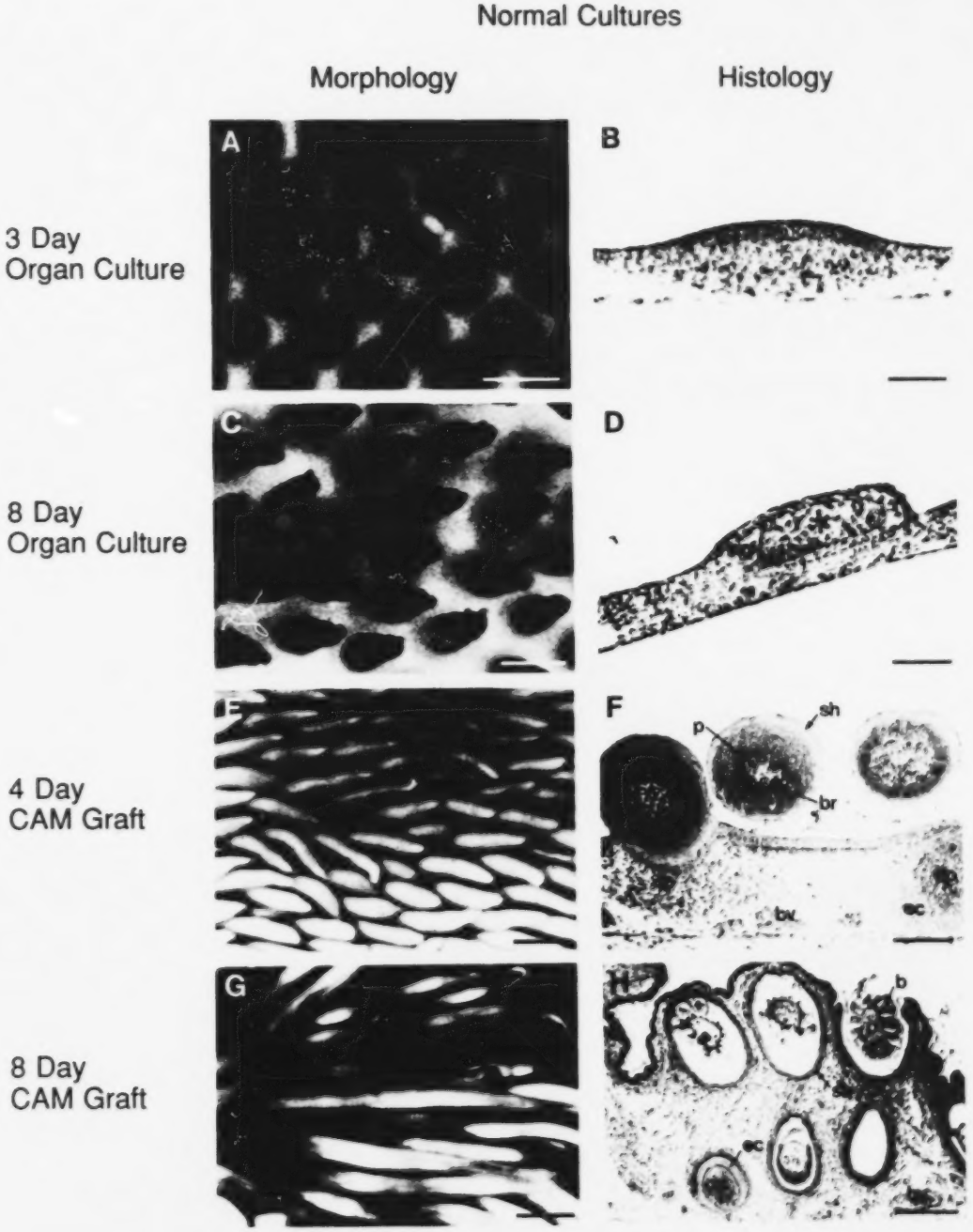
Skin explanted from stage 30 (E7) embryos and cultured for 3 days in medium alone underwent normal development of feather rudiments, as described previously (Brotman, 1980; Davidson, 1983a, b). Specifically, rudiments formed in a regularly spaced, hexagonal pattern (Fig. III-1A), and each rudiment consisted of a condensation with a dense population of dermal cells, overlaid by a placode of epidermal cells (Fig. III-1B). The rudiments were clearly visible as thickened areas of the skin surrounded by areas of decreased thickness and cell density (Figs. III-1A and B).

Culturing the stage 30 (E7) skin explants for 8 days led to the formation of a short, filament-like structure (FLS) from each of the rudiments (Fig. III-1C), as described previously (Gallin et al., 1986). Each FLS consisted of a thick bud bent over in an anterioposterior direction (Fig. III-1C). Sagittal sections showed that each FLS consisted of an epidermal layer (Fig. III-1D, margin indicated by dotted line) and a central core of dermis (indicated by *). There was no evidence for the onset of barb ridge formation in the epidermis of an FLS. Morphologically and histologically, each FLS resembled a young feather filament in vivo at stage 37 (E11) (Lucas and Stettenheim, 1972; Chuong and Edelman, 1985a).

ChAM grafting the 3-day organ cultures and allowing the grafts to grow for 4 days led to elongation of the feather rudiments and to formation of filaments that resembled stage 39 (E13) filaments in vivo (Fig. III-1E) (Lucas and Stettenheim, 1972; Chuong and Edelman, 1985a). Sections of a 4-day filament (Fig. III-1F) showed that each filament had a sheath (sh), barb ridges (br), and a central core of pulp (p) (for reference see Fig. I-7, Part B, a). In the dermis,

Figure III-1. Morphology and histology of normal 3-day and 8-day organ cultures and 4-day and 8-day CAM grafts. Dorsal skins were removed from stage 30 (E7) chicken embryos and allowed to grow for 3 days (A-B) or 8 days (C-D) in culture to examine the development of feather rudiments in vitro. Parallel 3-day cultures were grafted onto chorioallantoic membranes of 10 day chicken embryos and allowed to grow for an additional 4 days (E-F) or 8 days (G-H) to examine the development of feather filaments in vitro. For morphology, cultures and grafts were stained with carmine borate and photographed using transmitted light (A and C) or reflected light (E and G). For histology, cross sections (B, F, and H) or sagittal sections (D) were stained with hematoxylin and eosin and photographed using bright field illumination. In (D), the dotted line represents the inner margin of the epidermis and * represents the central core of dermis. In (F) and (H), b = barb, br = barb ridge, bv = blood vessel, dp = dermal papilla, ec = epidermal collar, p = pulp, and sh = sheath. Wherever possible, cultures and grafts are oriented such that cephalic is to the left and caudal is to the right. Scale bars are 500 μm (A, C, E, and G), 50 μm (B and D), 100 μm (F), and 200 μm (H).

Normal Cultures



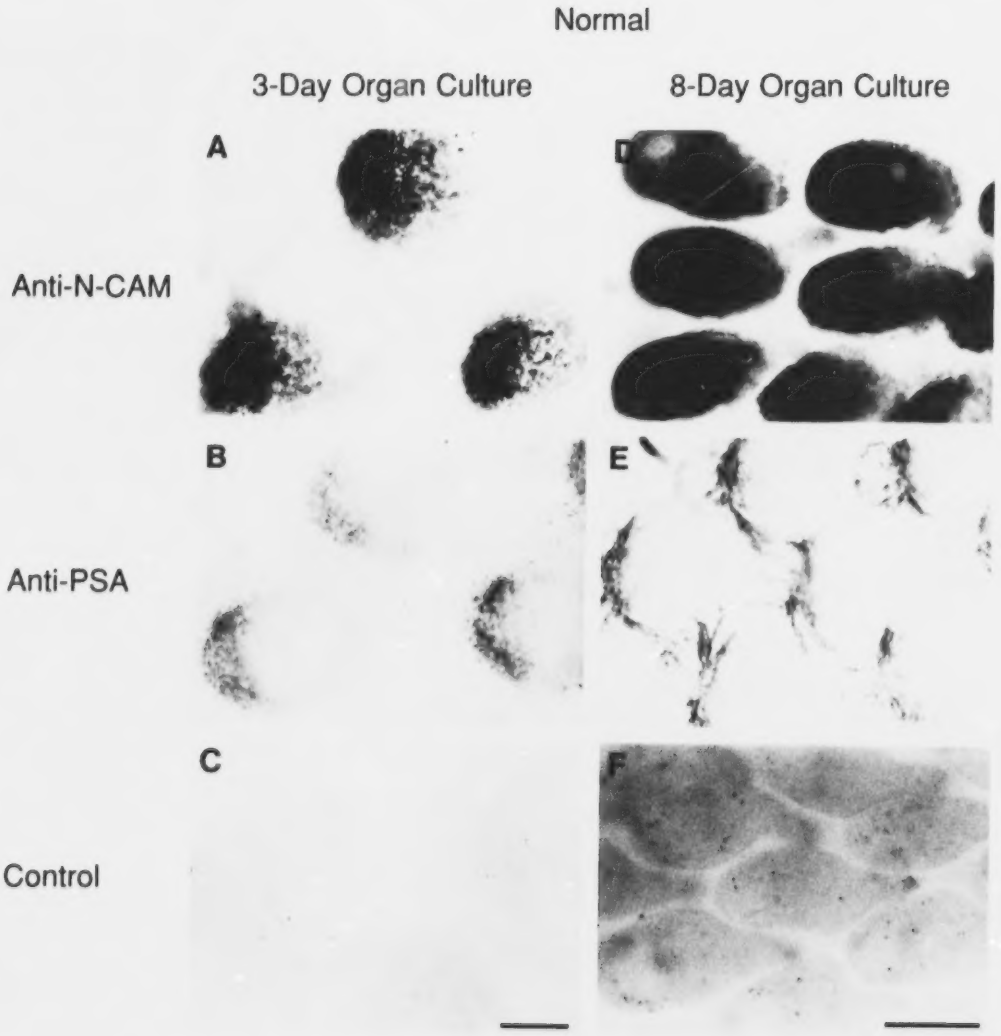
dermal papillae (dp) were visible as regions of high cell density surrounded by regions of lower cell density, and an epidermal collar (ec) was present around each papillae. Blood vessels (bv) were also present within the dermis of the graft.

Allowing the 4-day ChAM grafts to grow for an additional 4 days led to formation of long, slender filaments in the 8-day grafts (Fig. III-1G) that resembled stage 42 (E16) filaments in vivo (Lucas and Stettenheim, 1972). Each filament had barbs (Fig. III-1H, b), a core of pulp (p), a sheath (sh), a dermal papilla (dp), and an epidermal collar (ec). The barbs appeared to be highly keratinized as shown by their rough outlines and the presence of thread-like material around them; barbs from stage 42 (E16) filaments in vivo are known to be keratinized (Haake et al., 1984) and do not section cleanly, producing similar thread-like material (Marsh, unpublished observation). Keratinized barbs do not adhere well to the slide during staining and, for this reason, some of the feather follicles appear not to contain any barbs (Fig. III-1H, lower right). Blood vessels (bv) were present within the dermis of the graft.

Expression of N-CAM and PSA in Normal Cultures and Grafts

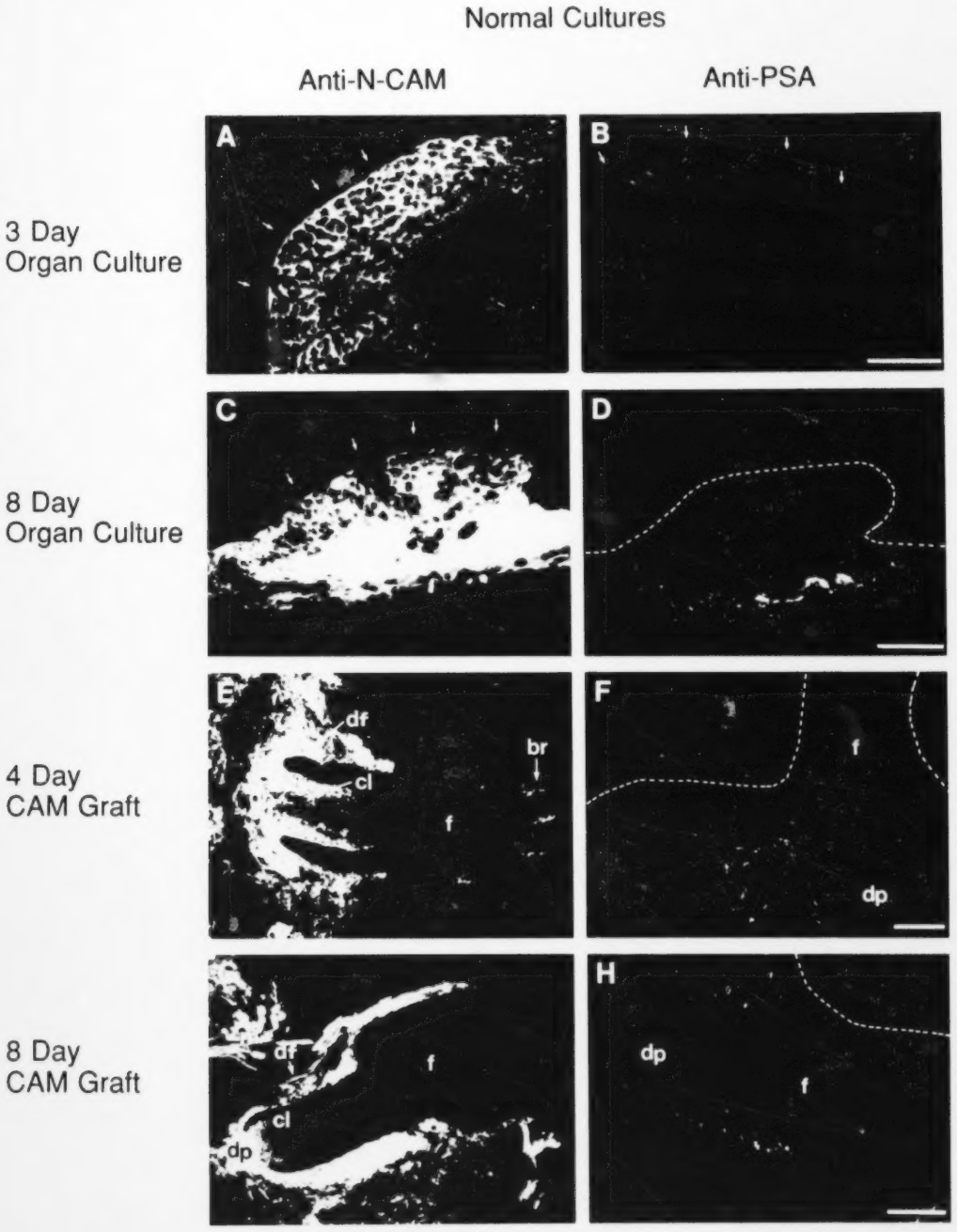
To ensure that the expression of N-CAM and PSA in the organ cultures and ChAM grafts was the same as in feather rudiments and filaments in vivo, the expression of these two molecules was examined in the cultures and grafts using immunoperoxidase staining of wholemounts (Fig. III-2) and immunofluorescent staining of sections (Fig. III-3).

Figure III-2. Immunoperoxidase staining of wholemounts of normal 3-day (A-C) and 8-day (D-F) organ cultures with anti-N-CAM 802 polyclonal antibody (A and D), anti-PSA 5A5 mAb (B and E), and an irrelevant mAb (C and F). In all cases, skins are oriented with cephalic to the left and caudal to the right. In the 3-day cultures, N-CAM and PSA are expressed more strongly in the cephalic region of the rudiments (A and B). In the 8-day cultures, N-CAM is expressed more strongly in the basal region of the filament-like structures (D), and PSA is detected only on cells in the dermis (E). Staining is not present in the controls (C and F). Scale bars are 200 μm.


Normal
3-Day Organ Culture
8-Day Organ Culture
A
D
Anti-N-CAM
B
E
Anti-PSA
C
F
Control

Localization of N-CAM and PSA in the 3-day organ cultures (Figs. III-2, A-C and III-3, A-B) showed that expression of these molecules in cultured rudiments was identical to expression of N-CAM and PSA in rudiments in vivo (Chuong and Edelman, 1985a; Marsh and Gallin, 1992). In particular, examination of N-CAM expression in the cultures over the 3 day culture period showed that N-CAM was initially expressed throughout epidermis and dermis (not shown) but became localized to cells of the rudiments as in vivo (Figs. III-2A and III-3A; see also Figs. II-2A, C, and E). PSA was not detected in the explants during the first 2 days in culture (not shown), but was expressed in the condensations after 3 days in culture (Fig. III-2B and III-3B). This correlated well with the first detectable expression of PSA in rudiments in vivo at stage 35 (E9) (Marsh and Gallin, 1992). Sections of the 3-day rudiments showed that N-CAM was present on cells of the dermal condensations, but N-CAM staining of the epidermal placodes was not seen (Fig. III-3A). N-CAM staining of the placodes was confirmed by immunoperoxidase staining of separated epidermis from the 3-day cultures (not shown; staining identical to Fig. II-2E), indicating that the absence of N-CAM staining in the sections was due to the plane of sectioning. PSA was expressed on cells of the condensations (Fig. III-3B), but was not detected in the epidermis of the sections (Fig. III-3B) or in separated epidermis (not shown). Both N-CAM and PSA were expressed more strongly in the cephalic region of the cultured rudiments (Fig. III-2, A-B), as in rudiments in vivo. Fig. III-2C represents a control culture stained with an irrelevant antibody.

Figure III-3. Immunofluorescent staining of sections of normal 3-day and 8-day organ cultures and 4-day and 8-day CAM grafts with anti-N-CAM 802 polyclonal antibody (A, C, E, and G) and anti-PSA 5A5 mAb (B, D, F, and H). In all cases, the arrows and dotted lines indicate the outer margin of the epidermis. In the 3-day organ cultures, both N-CAM and PSA are present in the dermal condensations (A-B; single rudiment is shown). In the 8-day organ cultures, N-CAM is expressed more strongly in the basal region of the dermis within the filament-like structure, and is also present on cells of the underlying dermis (C). In contrast, PSA is restricted to cells in the dermis (D). In the 4-day CAM grafts, N-CAM is present on cells of the dermal papilla (dp), epidermal collar (cl), dermal cells of the follicle (df), and cells of the barb ridges (br) in the filament (f) (E). PSA is restricted to cells in the dermis around the base of the feather follicle (F). The position of the dermal papilla (dp) and filament (f) are shown for orientation. In the 8-day CAM grafts, N-CAM is present on cells of the dermal papilla (dp), dermal cells of the follicle (df), and on muscle fibers in the dermis (m), but is expressed only faintly on cells of the epidermal collar (cl) and is no longer present in the filament (f) (G). PSA is restricted to cells in the dermis around the base of the feather follicle (H). The position of the dermal papilla (dp) and filament (f) are shown for orientation. Scale bars are 50 μm (A-D) and 100 μm (E-H).


Normal Cultures
Anti-N-CAM
Anti-PSA
3 Day
Organ Culture
8 Day
Organ Culture
4 Day
CAM Graft
8 Day
CAM Graft
A
B
C
D
E
F
H
df
cl
br
f
dp

Expression of N-CAM and PSA in the 8-day cultures (Figs. III-2, D-E and III-3, C-D) resembled staining of these molecules in stage 37 (E11) filaments in vivo (Chuong and Edelman, 1985a). N-CAM was not present on cells of the epidermis, but was expressed on dermal cells within each FLS and on cells of the underlying dermis (Fig. III-3C). The expression of N-CAM was stronger in the basal portion of each FLS (Fig. III-2D and III-3C). Although not visible in the wholemount (Fig. III-2D, out of plane of focus), N-CAM was present on bands of cells radiating between the rudiments (identical to PSA staining in Fig. III-2E). Expression of PSA was restricted to cells in the dermis (Fig. III-3D). Wholemount staining showed that PSA, like N-CAM, was present on bands of cells radiating between the rudiments (Figs. III-2E). This staining pattern resembled the staining of smooth muscle cells of feather erector muscle in vivo (see Fig. II-3I), but appeared somewhat less organized. Wholemount staining with an anti-α-actin mAb (not shown) confirmed that the N-CAM and PSA expressing cells in the dermis were smooth muscle. Fig. III-2F represents an 8-day culture stained with an irrelevant antibody.

Expression of N-CAM and PSA in the 4-day ChAM grafts resembled staining of these molecules in stage 39 (E13) filaments in vivo (Chuong and Edelman, 1985a). N-CAM was expressed on cells of the dermal papilla (dp), dermal cells of the follicle (df), cells of the epidermal collar (cl), and on cells of the barb ridges (br) within the filament (f) (Fig. III-3E). Staining of wholemounts (not shown) confirmed that N-CAM was expressed on cells of the barb ridges in

the filaments and also showed that N-CAM was present on a criss-cross pattern of cells within the dermis (similar to Fig. II-3I). Expression of PSA in the 4-day ChAM grafts was restricted to cells within the dermis (Fig. III-3F). Wholemount staining revealed that PSA was also expressed on a criss-cross pattern of cells in the dermis (not shown). Staining with the anti-α-actin mAb showed that the N-CAM and PSA expressing cells in the dermis were smooth muscle (not shown).

Expression of N-CAM and PSA in the 8-day ChAM grafts was similar to staining of these molecules in stage 42 (E16) filaments in vivo (Chuong and Edelman, 1985a). Specifically, N-CAM was expressed on cells of the dermal papilla (dp), dermal cells of the follicle (df), and on muscle fibers (m) present within the dermis (Fig. III-3G). N-CAM was not expressed on cells within the filament (f), and was weakly expressed on cells of the collar (cl) (Fig. III-3G). Wholemount staining (not shown) confirmed that N-CAM was not expressed in the filaments, and showed that N-CAM was present on a criss-cross pattern of cells within the dermis, as in the 4-day ChAM grafts. Expression of PSA in the 8-day grafts was restricted to cells within the dermis (Fig. III-3H). Wholemount staining (not shown) revealed that PSA was also present on a criss-cross pattern of cells within the dermis. Staining with the anti-α-actin mAb confirmed that the N-CAM and PSA expressing cells in the dermis were smooth muscle (not shown).

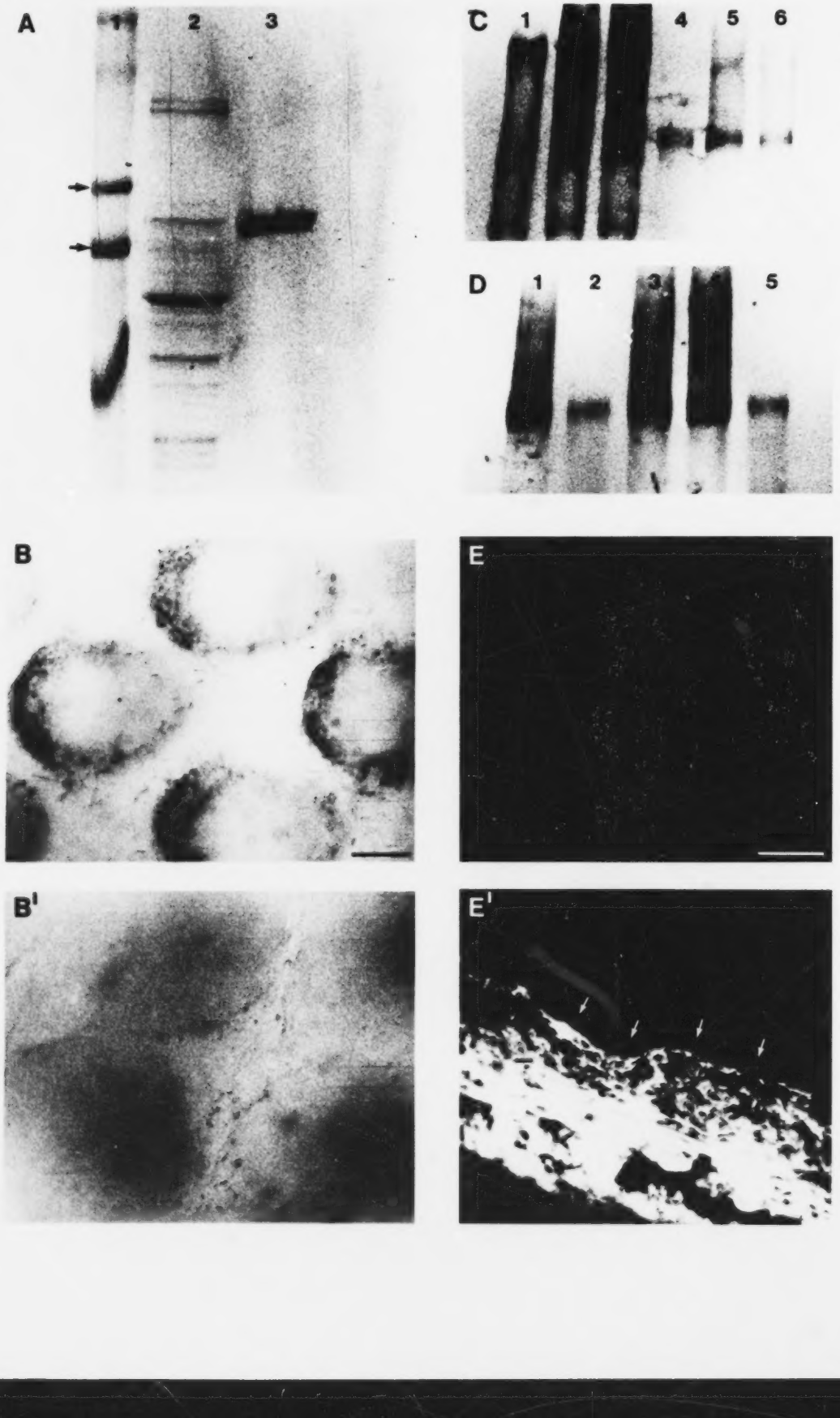
Purification and Activity of Endo-N, and Ability of CAM6 mAb to Penetrate Tissue

Having determined the morphology, histology, and expression of N-CAM and PSA in normal cultures and grafts, the next step was to find out what changes in these parameters occurred upon perturbing N-CAM-mediated adhesion in these cultures and grafts using (a) endo-N to remove PSA, (b) a monoclonal antibody (CAM6 mAb) to inhibit N-CAM homophilic adhesion and, (c) both reagents together.

In order to obtain the large quantities of endo-N required for the perturbations, endo-N was purified from lysates of phage-infected *E. coli* (Fig. III-4A). Numerous bacterial and phage proteins were present in the crude bacterial lysate (Fig. 4A, lane 2), but only a single 105 kD band was present in the purified endo-N stock (Fig. 4A, lane 3; standards shown in lane 1). The size of this band corresponds to the known size of endo-N (Hallenbeck et al., 1987). Endo-N is optimally active at physiological pH (pH 7.2) and specifically digests PSA (Vimr et al., 1984; Hallenbeck et al., 1987). Previous studies have shown that all detectable PSA in the chicken embryo is associated with N-CAM (Vimr et al., 1984; Rutishauser et al., 1985).

The objective of the endo-N perturbation was to determine what changes in rudiment and filament development occurred upon removal of PSA from N-CAM in skin. It was therefore necessary to find out what concentration of endo-N was sufficient to completely remove the PSA from skin N-CAM. Concentrations of endo-N used in previous perturbation experiments (Rutishauser et al., 1985; Acheson

Figure III-4. Purification of endoneuraminidase-N (endo-N), activity of endo-N, and penetration of CAM6 mAb into tissue. (A) Commassie blue stained polyacrylamide gel showing the purification of endo-N from bacterial lysate. Multiple bands are present in the crude bacterial lysate (lane 2), but only a single 105 kD band is present in the purified endo-N stock (lane 3). Upper and lower arrow (lane 1) indicate 116 kD and 97 kD protein standards, respectively. (B and B') Immunoperoxidase staining of PSA in untreated (B) and 50 U/ml endo-N treated (B') 3-day organ cultures, showing this concentration of endo-N is sufficient to completely remove PSA from the feather rudiments. Scale bar is 100 μm. (C) Digestion of affinity purified brain N-CAM (lanes 1-3) and affinity-purified skin N-CAM (lanes 4-6) with 50 U/ml endo-N (lanes 1 and 4), 50 U/ml boiled endo-N (lanes 2 and 5), and with no enzyme (lanes 3 and 6). In the boiled endo-N and untreated samples (lanes 2, 3, 5, and 6), the dark smearing reflects the presence of PSA on the N-CAM. PSA is removed by digestion with endo-N, as shown by the absence of smearing (lanes 1 and 4). (D) Western blot analysis of N-CAM in untreated (lane 1), 50 U/ml endo-N (lane 2), 50 U/ml boiled endo-N (lane 3), 125 μg/ml CAM6 mAb (lane 4), and 50 U/ml endo-N plus 125 μg/ml CAM6 mAb (lane 5) treated 3-day organ cultures. PSA is removed in cultures treated with active endo-N (lanes 2 and 5), as shown by the absence of smearing. The amount of N-CAM present in these cultures appears decreased compared to the amount of N-CAM in the samples not treated with active endo-N (compare lanes 2 and 5 to lanes 1, 3, and 4). Similar amounts of protein were run in each lane. (E and E') Immunofluorescent staining of sections of untreated (E) and 125 μg/ml CAM6 mAb treated (E') 3-day organ cultures with rabbit anti-mouse FITC-conjugated antibody only. The intense staining in (E') reflects the presence of CAM6 mAb in the tissue, and indicates that the antibody is able to penetrate and disperse in the skin explants. Staining is present in the epidermis (outer margin indicated by arrows), but the dermal staining is so intense that epidermal staining appears absent. Scale bar is 50 μm.


A
1
2
3
C
1
4
5
6
D
1
2
3
5
B
E
B'
E'

and Rutishauser, 1988; Landmesser et al., 1990) ranged from 25-50 U/ml. To determine what concentration of endo-N was required to remove PSA from skin N-CAM, 3-day cultures were grown in medium containing a range of endo-N concentrations from 20-70 U/ml, and the expression of PSA in the rudiments was monitored by immunoperoxidase staining. PSA was detected in the rudiments of untreated 3-day cultures (Fig. III-4B) but was absent in the rudiments of 50 U/ml endo-N treated 3-day cultures (Fig. III-4B'). Immunofluorescent staining of untreated and 50 U/ml treated cultures gave identical results to the immunoperoxidase staining (not shown). Skin explants treated with a lower concentration of endo-N (40 U/ml) still showed faint PSA staining (not shown). For this reason, all of the endo-N perturbations were done using 50 U/ml endo-N.

To ensure that the absence of PSA staining in the endo-N treated cultures was due to digestion of PSA by the endo-N and not to other factors such as toxicity, the ability of the purified endo-N to remove PSA from affinity-purified brain and filament N-CAM was tested (Fig. III-4C), and removal of PSA from N-CAM in the cultures was monitored by Western blot analysis (Fig. III-4D). Digestion with endo-N removed PSA from both brain (Fig. III-4C, lane 1) and filament (lane 4) N-CAM, as shown by the decreased smearing compared to undigested brain and filament N-CAM (lanes 2 and 5) and to brain and filament N-CAM digested with boiled endo-N (denatured by heating 10 min at 100°C) (lanes 3 and 6). Western blot analysis of N-CAM in the 3-day organ cultures (Fig. III-4D) showed that PSA was present on N-CAM in untreated (lane 1), boiled

endo-N treated (lane 3), and CAM6 mAb treated (lane 3) cultures, but was removed from N-CAM in the endo-N treated (lane 2) and endo-N plus CAM6 mAb treated (lane 5) cultures. Interestingly, the amount of N-CAM present in the cultures treated with active endo-N (Fig. III-4D, lanes 2 and 5) appeared to be less than the amount of N-CAM present in the untreated (lane 1), boiled endo-N treated (lane 3), and CAM6 mAb treated (lane 4) cultures.

The presence of PSA on N-CAM in untreated and treated 8-day organ cultures and 4-day and 8-day ChAM grafts was also examined by Western blot analysis to (a) verify that PSA was still absent in the endo-N treated cultures and grafts and, (b) to determine if a decreased amount of N-CAM was present in the cultures and grafts treated with endo-N. An identical staining pattern to that shown in Fig. III-4D was seen on Western blots of N-CAM from the 8-day cultures and the 4-day and 8-day grafts (not shown).

The objective of the antibody perturbation experiments was to determine what changes in rudiment and filament development occurred upon altering N-CAM homophilic binding. Using a vesicle aggregation assay, anti-N-CAM mAb CAM6 was found to inhibit N-CAM-mediated aggregation of brain vesicles by 80% (see Materials and Methods). This suggested that CAM6 would be suitable for the perturbation experiments. However, unlike the perturbation using endo-N, where the concentration of enzyme required to remove the PSA was readily determined, there was no simple way to determine the concentration of CAM6 required to inhibit N-CAM binding in the skin cultures. Previous perturbation experiments used concentrations of mAb between 10-100 μg/ml (Fraser et al., 1984; Keane et

al., 1988; Aoki et al., 1991). Based on this information, the effects of a range of CAM6 mAb concentrations (10-250 µg/ml) on morphology of 3-day and 8-day cultures and 4-day and 8-day grafts was initially tested. No change in the morphology of the cultures or grafts was apparent at even the highest concentration of CAM6 mAb tested (not shown). In the aggregation assay, the concentration of CAM6 mAb was about 125 µg/ml. This concentration was shown to retard N-CAM-mediated aggregation of brain vesicles by 80%. Because embryonic brain contains much more N-CAM than embryonic skin (based on immunofluorescent staining and Western blot analysis), this concentration of CAM6 mAb should be sufficient to inhibit the majority of N-CAM homophilic binding in the cultures. For this reason, this concentration of CAM6 mAb was used for the antibody perturbation experiments.

To ensure that the observed lack of effect of the CAM6 mAb on rudiment and filament development was not due to inability of the antibody to penetrate into the tissue, untreated and 125 µg/ml CAM6 mAb treated 3-day organ cultures were sectioned and stained with FITC-conjugated rabbit anti-mouse antibody only (Fig. III-4, E and E'). No staining was detected in the untreated 3-day skin culture (Fig. III-4E). In contrast, intense staining was seen throughout the CAM6 mAb treated culture (Fig. III-4E'). Staining was present in both the epidermis and dermis, but the dermal staining was so strong that the epidermal staining appears absent (Fig. III-4E'). The lack of effect of the CAM6 mAb on rudiment and filament development was therefore not due to inability of the mAb to penetrate and disperse in the skin.

Morphology and Histology of Endo-N and CAM6 Treated Cultures and Grafts

Removal of PSA using 50 U/ml endo-N and perturbation of N-CAM homophilic binding using 125 μg/ml CAM6 mAb did not lead to any discernable changes in morphology or histology of the organ cultures or ChAM grafts (Figs. III-5 and III-6) compared to the untreated controls (Fig. III-1). As well, no differences were seen between the untreated controls and boiled endo-N treated cultures and grafts (not shown).

In the endo-N and CAM6 treated 3-day organ cultures, rudiments formed in a regular, hexagonal pattern (Figs. III-5A and III-6A), as in the untreated controls (Fig. III-1A). Comparison of rudiment diameter in the treated and untreated cultures using the Student's t-test showed that there was no significant difference ($p \geq 0.05$) in this parameter between the endo-N treated (355 μm ± 28, n=20), CAM6 mAb treated (371 μm ± 34, n=20), and untreated cultures (361 μm ± 22, n=20). Likewise, center to center distance between rudiments was not significantly different ($p \geq 0.05$) in the endo-N treated (458 μm ± 29, n=20), CAM6 mAb treated (461 μm ± 31, n=20), and untreated (469 μm ± 24, n=20) cultures. Thus, the dimensions of the hexagonal rudiment pattern were not altered by the perturbations. The rudiments in both the endo-N and CAM6 mAb treated cultures consisted of a defined dermal condensation with a dense population of cells, overlaid by an epidermal placode (Figs. III-5B and III-6B). Individual rudiments were discernable as thickened areas of the skin surrounded by areas of decreased thickness and cell density (Figs. III-5A and III-6A), as in the untreated controls (Fig. III-1, A and B).

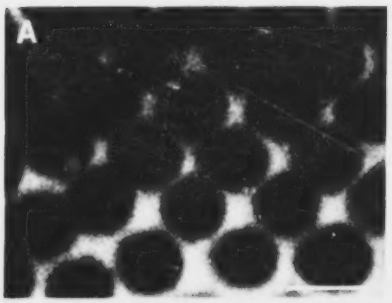
Figure III-5. Morphology and histology of 50 U/ml endo-N treated 3-day and 8-day organ cultures and 4-day and 8-day CAM grafts. Skins were removed from stage 30 (E7) chicken embryos and allowed to grow for 3 days (A-B) or 8 days (C-D) in culture to examine the effect that removing PSA has on the development of feather rudiments. Parallel 3-day cultures were grafted onto chorioallantoic membranes of 10 day chicken embryos and allowed to grow for an additional 4 days (E-F) or 8 days (G-H) to examine the effect of removing PSA on the development of feather filaments. No differences in the morphology and histology of the endo-N treated cultures and grafts were seen compared to the untreated controls (Fig. III-1). Skins were processed and photographed as in Fig. III-1. In (D), the dotted line represents the inner margin of the epidermis and * represents the central core of dermis. In (F) and (H), b = barb, br = barb ridge, bv = blood vessel, dp = dermal papilla, ec = epidermal collar, p = pulp, and sh = sheath. Wherever possible, cultures and grafts are oriented such that cephalic is to the left and caudal is to the right. Scale bars are 500 μm (A, C, E, and G), 50 μm (B and D), 100 μm (F), and 200 μm (H).

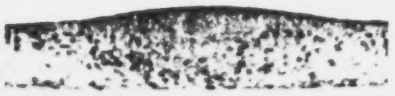
50 U/mL Endo-N Treated Cultures

Morphology

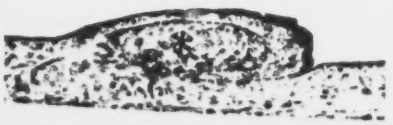
Histology

3 Day
Organ Culture

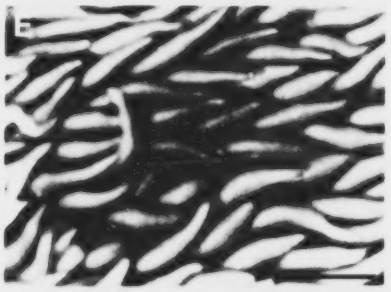


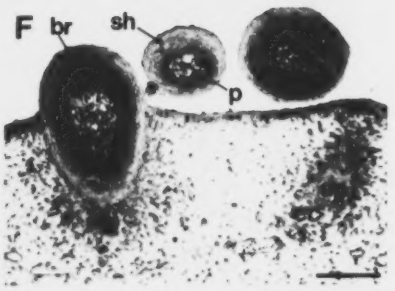


8 Day
Organ Culture



4 Day
CAM Graft





8 Day
CAM Graft

In the endo-N and CAM6 mAb treated 8-day organ cultures, short, filament-like structures (FLS) developed from each rudiment (Figs. III-5C and III-6C), as described for the untreated cultures (Fig. III-1C). Each FLS consisted of an epidermal layer and a core of dermis, and there was no evidence for barb ridge formation in the epidermis (Figs. III-5D and III-6D). The length of the FLS in the endo-N treated (472 μm ± 26, n=20), CAM6 mAb treated (477 μm ± 24, n=20), and untreated cultures (464 μm ± 32, n=20) was not significantly different ($p \geq 0.05$), indicating that growth of the FLS was not inhibited by the perturbations.

As with the 3-day and 8-day cultures, the endo-N and CAM6 mAb treated 4-day ChAM grafts appeared identical in both morphology (Figs. III-5E and III-6E) and histology (Figs. III-5F and III-6F) to the untreated controls (Fig. III-1, E-F). Elongation of the feather rudiments into filaments occurred (Figs. III-5E and III-6E), and each filament had a sheath (sh), barb ridges (br), pulp (p), dermal papillae (dp), and an epidermal collar (Figs. III-5F and III-6F). The length of the filaments in the endo-N treated (992 μm ± 55, n=20), CAM6 mAb treated (977 μm ± 38, n=20), and untreated ChAM grafts (968 μm ± 46, n=20) was not statistically different ($p \geq 0.05$), indicating that growth of the filaments was not retarded by the perturbations.

Endo-N and CAM6 mAb treated 8-day ChAM grafts likewise had the same morphology (Figs. III-5G and III-6G) and histology (Figs. III-5H and III-6H) as the untreated controls (Fig. III-1, G-H). In particular, long slender filaments formed (Fig. III-5G and III-6G), and each filament had barbs (b), pulp (p), sheath (sh), dermal papilla (dp), and epidermal collar (ec) (Fig. III-5H and III-6H). As in the

Figure III-6. Morphology and histology of 125 μg/ml CAM6 mAb treated 3-day and 8-day organ cultures and 4-day and 8-day CAM grafts. Skins were removed from stage 30 (E7) chicken embryos and allowed to grow for 3 days (A-B) or 8 days (C-D) in culture to examine the effect that perturbing N-CAM homophilic binding has on the development of feather rudiments. Parallel 3-day cultures were grafted onto chorioallantoic membranes of 10 day chicken embryos and allowed to grow for an additional 4 days (E-F) or 8 days (G-H) to examine the effect of perturbing N-CAM homophilic binding on the development of feather filaments. No differences in the morphology and histology of the CAM6 mAb treated cultures and grafts was seen compared to the untreated controls (Fig. III-1). Skins were processed and photographed as in Fig. III-1. In (D), * represents the central core of dermis. In (F) and (H), b = barb, br = barb ridge, bv = blood vessel, dp = dermal papilla, ec = epidermal collar, p = pulp, and sh = sheath. Wherever possible, cultures and grafts are oriented such that cephalic is to the left and caudal is to the right. Scale bars are 500 μm (A, C, E, and G), 50 μm (B and D), 100 μm (F), and 200 μm (H).

125 µg/mL CAM6 Treated Cultures

Morphology

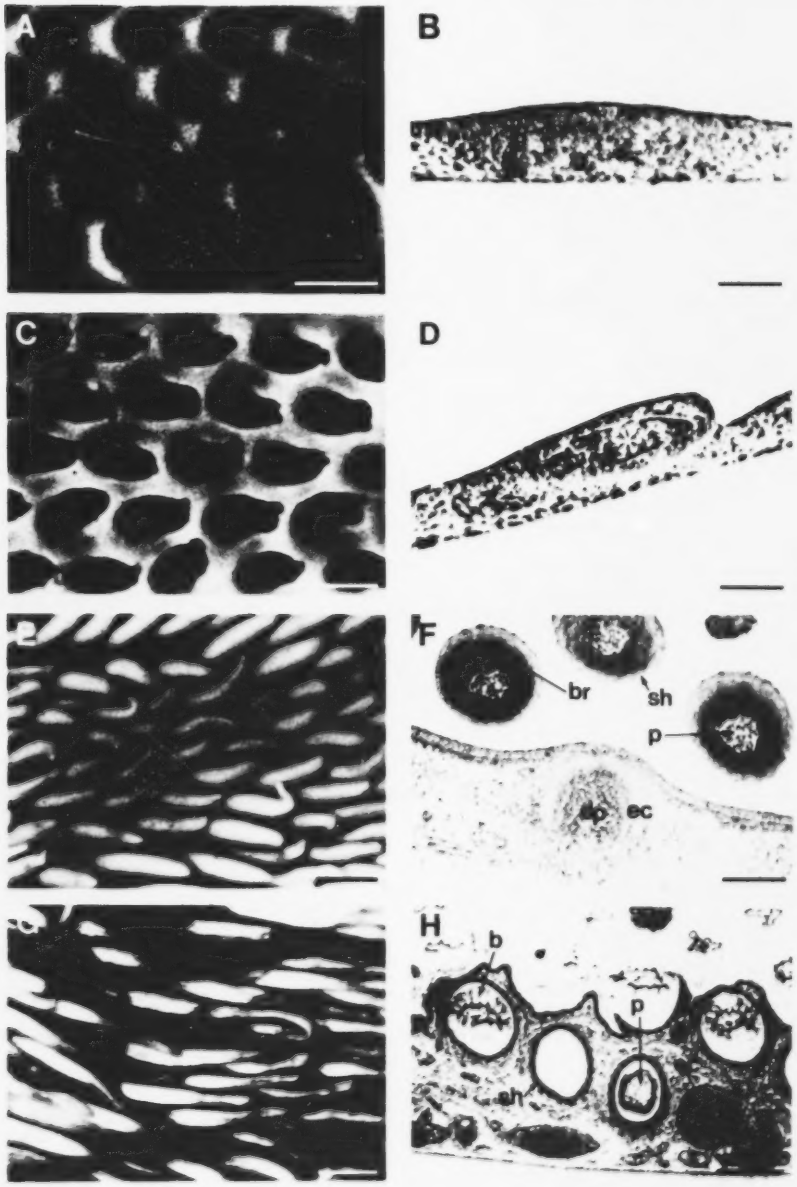
Histology

3 Day
Organ Culture

8 Day
Organ Culture

4 Day
CAM Graft

8 Day
CAM Graft



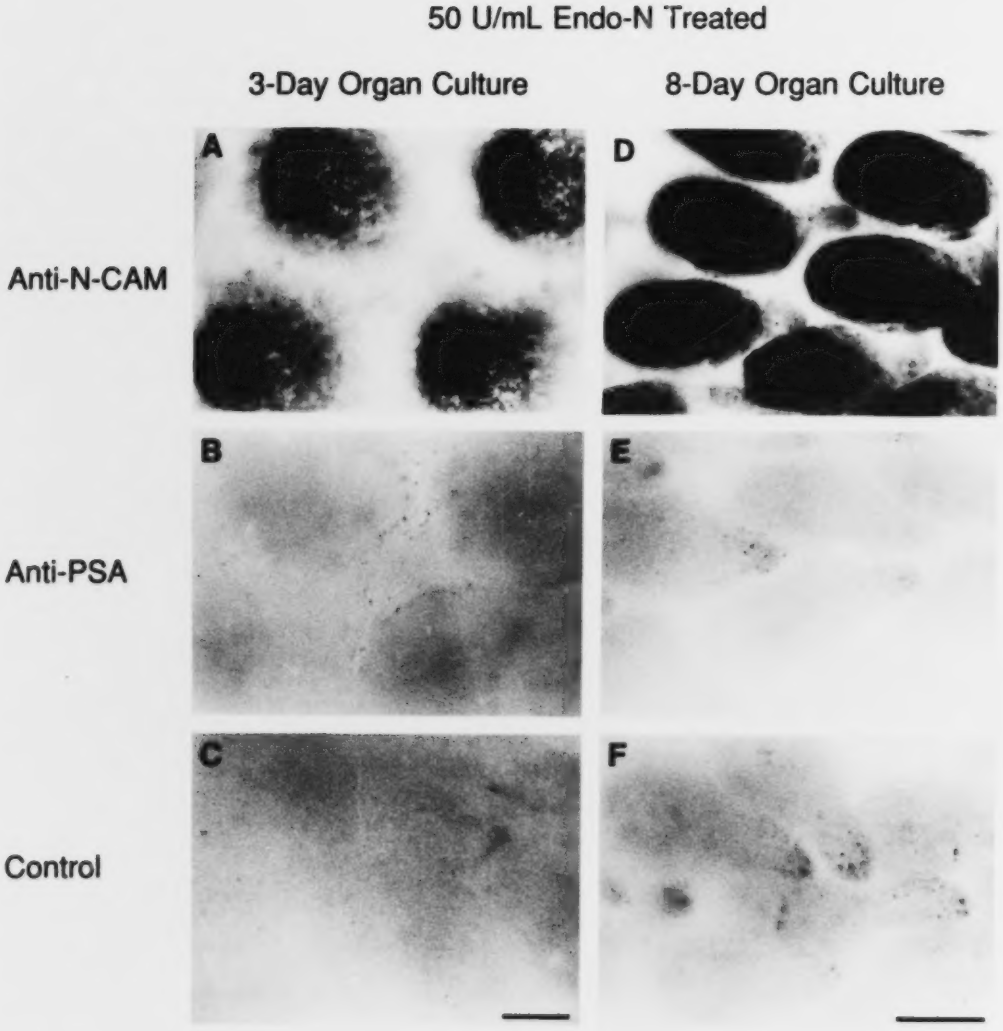
untreated 8-day grafts, the barbs in the perturbed grafts appeared to be keratinized (Fig. III-5H and III-6H) by virtue of their coarse appearance and the presence of thread-like material. The length of the filaments in the endo-N treated (2.5 mm ± 0.33, n=20), CAM6 mAb treated (2.3 mm ± 0.37, n=20), and untreated grafts (2.4 mm ± 0.42, n=20) was not statistically different ($p \geq 0.05$), indicating that growth of the filaments was not inhibited by the perturbations.

Because no effect on the morphology and histology of the organ cultures and ChAM grafts was seen with either the endo-N or CAM6 mAb perturbation, the effect of treatment with both reagents combined was also examined. As with the endo-N and CAM6 mAb perturbations alone, no detectable changes in the morphology and histology of the cultures and grafts occurred with the combined 50 U/ml endo-N and 125 µg/ml CAM6 mAb treatment (not shown).

Expression of N-CAM and PSA in Endo-N and CAM6 Treated Cultures and Grafts

Localization of N-CAM and PSA in wholemounts (Figs. III-7 and III-8) and sections (Figs. III-9 and III-10) of endo-N and CAM6 mAb treated 3-day organ cultures showed that the expression of these molecules was the same as the expression of N-CAM and PSA in control 3-day cultures (Figs. III-2 and III-3), except that PSA was completely removed by the endo-N treatment. In particular, N-CAM was localized to cells of the feather rudiments and was expressed predominantly in their cephalic region (Figs. III-7A and III-8A). PSA was also expressed in the cephalic region of the rudiments in CAM6 mAb treated cultures (Fig. III-8B), but was not detected in

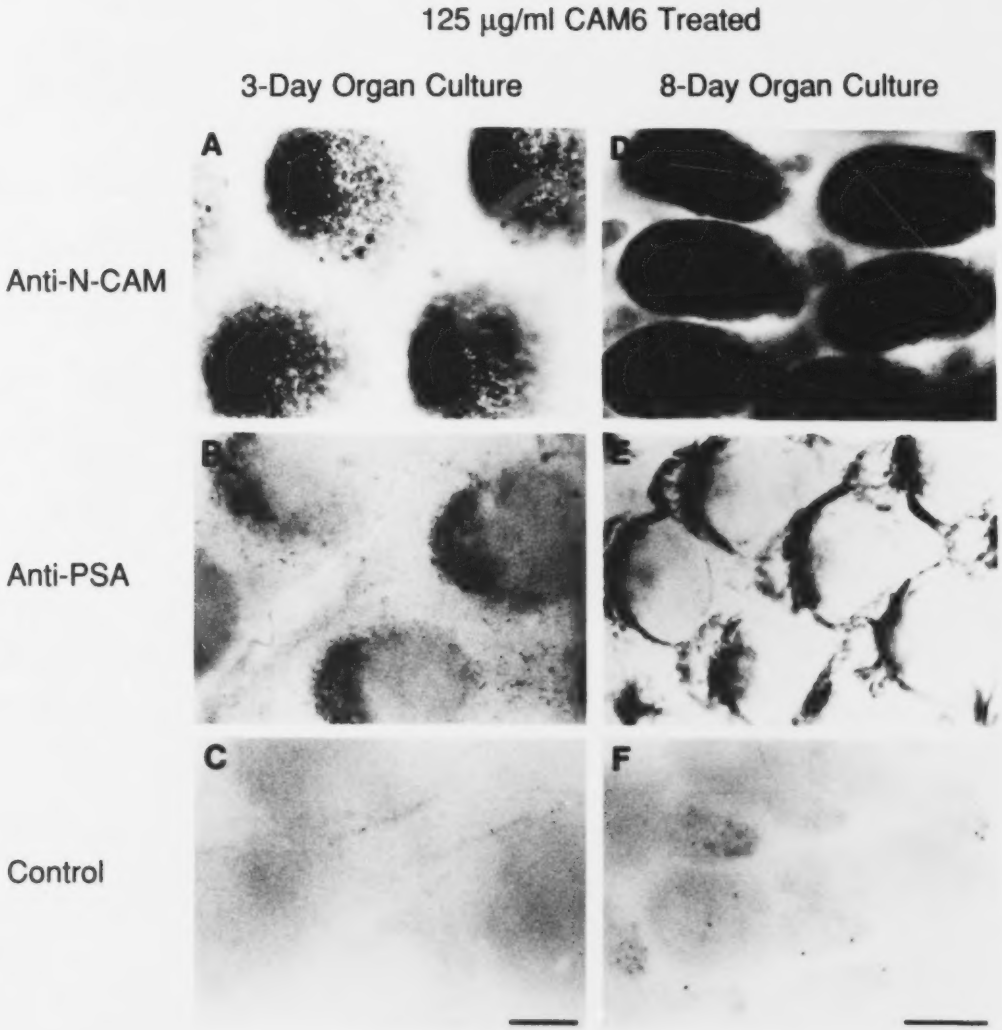
Figure III-7. Immunoperoxidase staining of wholemounts of 50 U/ml endo-N treated 3-day (A-C) and 8-day (D-F) organ cultures with anti-N-CAM 802 polyclonal antibody (A and D), anti-PSA 5A5 mAb (B and E), and an irrelevant mAb (C and F). In all cases, skins are oriented with cephalic to the left and caudal to the right. In the 3-day cultures, N-CAM is expressed more strongly in the cephalic region of the rudiments (A). PSA is not detected in the rudiments (B). In the 8-day cultures, N-CAM is expressed more strongly in the basal region of the filament-like structures (D). PSA is not detected on cells in the dermis (E). Staining is not detected in the controls (C and F). Scale bars are 200 μm.


50 U/mL Endo-N Treated
3-Day Organ Culture
8-Day Organ Culture
Anti-N-CAM
Anti-PSA
Control
A
B
C
D
E
F

rudiments of the endo-N treated cultures (Fig. III-7B). Staining of sections of the endo-N and CAM6 mAb treated cultures showed that N-CAM was expressed on cells of the dermal condensations. As in the untreated cultures (Fig. III-3A), N-CAM was not detected on cells of the epidermal placodes in sections of the treated cultures (Figs. III-9A and III-10A), but N-CAM staining of the placodes was present in immunoperoxidase-stained epidermis from both endo-N and CAM6 mAb treated cultures (not shown). PSA was expressed in condensations of the CAM6 mAb treated cultures (Fig. III-10B), but was not present in condensations of the endo-N treated cultures (Fig. III-9B). PSA was not detected in the epidermis of either the endo-N or CAM6 mAb treated cultures. Figures III-7C and III-8C represent endo-N and CAM6 mAb treated 3-day cultures that were stained with an irrelevant antibody.

As in the 3-day cultures, the expression of N-CAM and PSA in the endo-N and CAM6 mAb treated 8-day cultures (Figs. III-7, 8, 9 and 10) resembled staining of these molecules in the 8-day untreated controls (Figs. III-2 and III-3), except that PSA was completely removed by the endo-N treatment. In particular, N-CAM was expressed on dermal cells within each FLS and also on cells of the underlying dermis, but was not detected on cells of the epidermis (Figs. III-9C and III-10C). As in the untreated 8-day cultures, expression of N-CAM was stronger in the basal portion of each FLS (Figs. III-9C and III-10C). In wholemounts of endo-N and CAM6 mAb treated 8-day cultures, N-CAM was present on bands of cells within the dermis (Figs. III-7D and III-8D, out of plane of focus; identical to Fig. III-8E). In CAM6 mAb treated cultures, PSA was

Figure III-8. Immunoperoxidase staining of wholemounts of 125 µg/ml CAM6 mAb treated 3-day (A-C) and 8-day (D-F) organ cultures with anti-N-CAM 802 polyclonal antibody (A and D), anti-PSA 5A5 mAb (B and E), and an irrelevant mAb (C and F). In all cases, skins are oriented with cephalic to the left and caudal to the right. In the 3-day cultures, N-CAM and PSA are expressed more strongly in the cephalic region of the rudiments (A and B). In the 8-day cultures, N-CAM is expressed more strongly in the basal region of the filament-like structures (D), and PSA is detected only on cells in the dermis (E). Staining is not present in the controls (C and F). Scale bars are 200 µm.

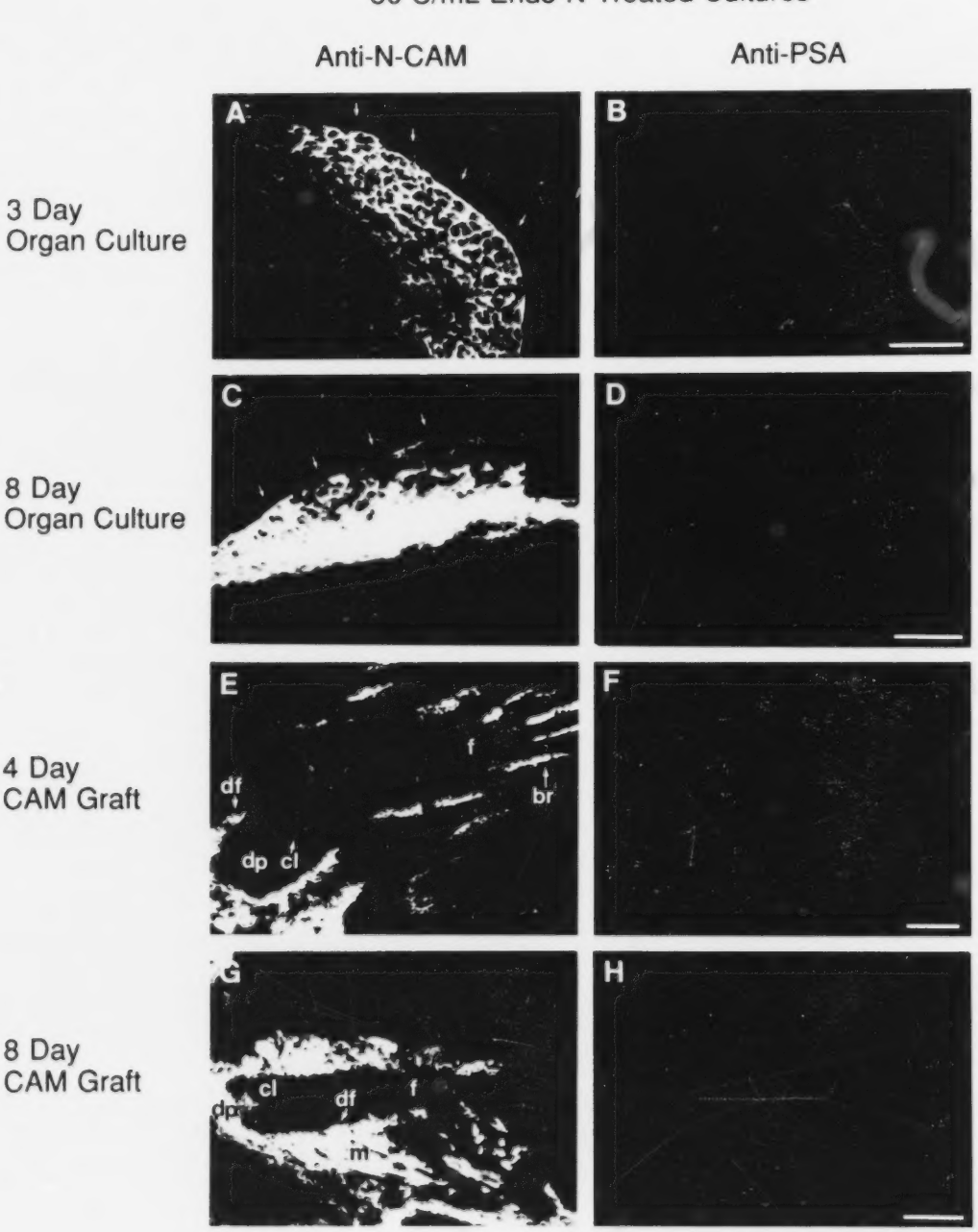

125 µg/ml CAM6 Treated
3-Day Organ Culture
8-Day Organ Culture
Anti-N-CAM
Anti-PSA
Control
A
D
B
E
C
F

expressed on cells in the dermis (Fig. III-10D), but PSA was not detected on these cells in the endo-N treated cultures (Fig. III-9D). Wholemount staining of PSA in CAM6 mAb treated cultures showed that PSA was present on bands of cells radiating between the bases of the filaments (Fig. III-8E). This staining was not detected in wholemounts of endo-N treated cultures (Fig. III-7E). Staining of wholemounts with the anti-α-actin mAb showed that the N-CAM and PSA expressing cells in the dermis were smooth muscle (not shown). Figures III-7F and III-8F represent endo-N and CAM6 mAb treated 8-day cultures stained with an irrelevant antibody.

As in the organ cultures, localization of N-CAM and PSA in endo-N and CAM6 mAb treated 4-day ChAM grafts (Figs. III-9 and III-10) showed that expression of these molecules was not altered by the perturbations, except that PSA was removed by the endo-N treatment. In both the endo-N and CAM6 mAb treated grafts, N-CAM was expressed on cells of the dermal papilla (dp), dermal cells of the follicle (df), cells of the epidermal collar (cl), and on cells of the barb ridges (br) within the filament (f) (Figs. III-9E and III-10E). Note that for Fig. III-9E, the section passed through the edge of the follicle and most of the dermal papilla and collar were therefore absent in this section. In the CAM6 mAb treated grafts, expression of PSA was restricted to cells in the dermis (Fig. III-10F). PSA staining was not detected in the endo-N treated grafts (Fig. III-9F). Wholemount staining of N-CAM in the endo-N and CAM6 mAb treated grafts showed that N-CAM was expressed on cells of the barb ridges within the filaments and on a criss-cross pattern of cells within the dermis (not shown), as described in the untreated controls. This same

Figure III-9. Immunofluorescent staining of sections of 50 U/ml endo-N treated 3-day and 8-day organ cultures and 4-day and 8-day CAM grafts with anti-N-CAM 802 polyclonal antibody (A, C, E, and G) and anti-PSA 5A5 mAb (B, D, F, and H). In all cases, the arrows and dotted lines indicate the outer margin of the epidermis. In the 3-day organ cultures, N-CAM is present in the dermal condensations (A; single rudiment is shown). PSA is not present in the condensations (B). In the 8-day organ cultures, N-CAM is expressed more strongly in the basal region of the dermis within the filament-like structure, and is also present on cells of the underlying dermis (C). PSA is not present in the 8-day cultures (D). In the 4-day CAM grafts, N-CAM is present on cells of the dermal papilla (dp), epidermal collar (cl), dermal cells of the follicle (df), and cells of the barb ridges (br) in the filament (f) (E). Note that this section is through the edge of the follicle and, for this reason, N-CAM staining on the dermal papilla and collar is absent or faint. PSA is not present in the 4-day CAM grafts (F). In the 8-day CAM grafts, N-CAM is present on cells of the dermal papilla (dp), dermal cells of the follicle (df), and on muscle fibers in the dermis (m), but is expressed only faintly on cells of the epidermal collar (cl) and is no longer present in the filament (f) (G). PSA is not present in the 8-day CAM grafts (H). Scale bars are 50 μm (A-D) and 100 μm (E-H).

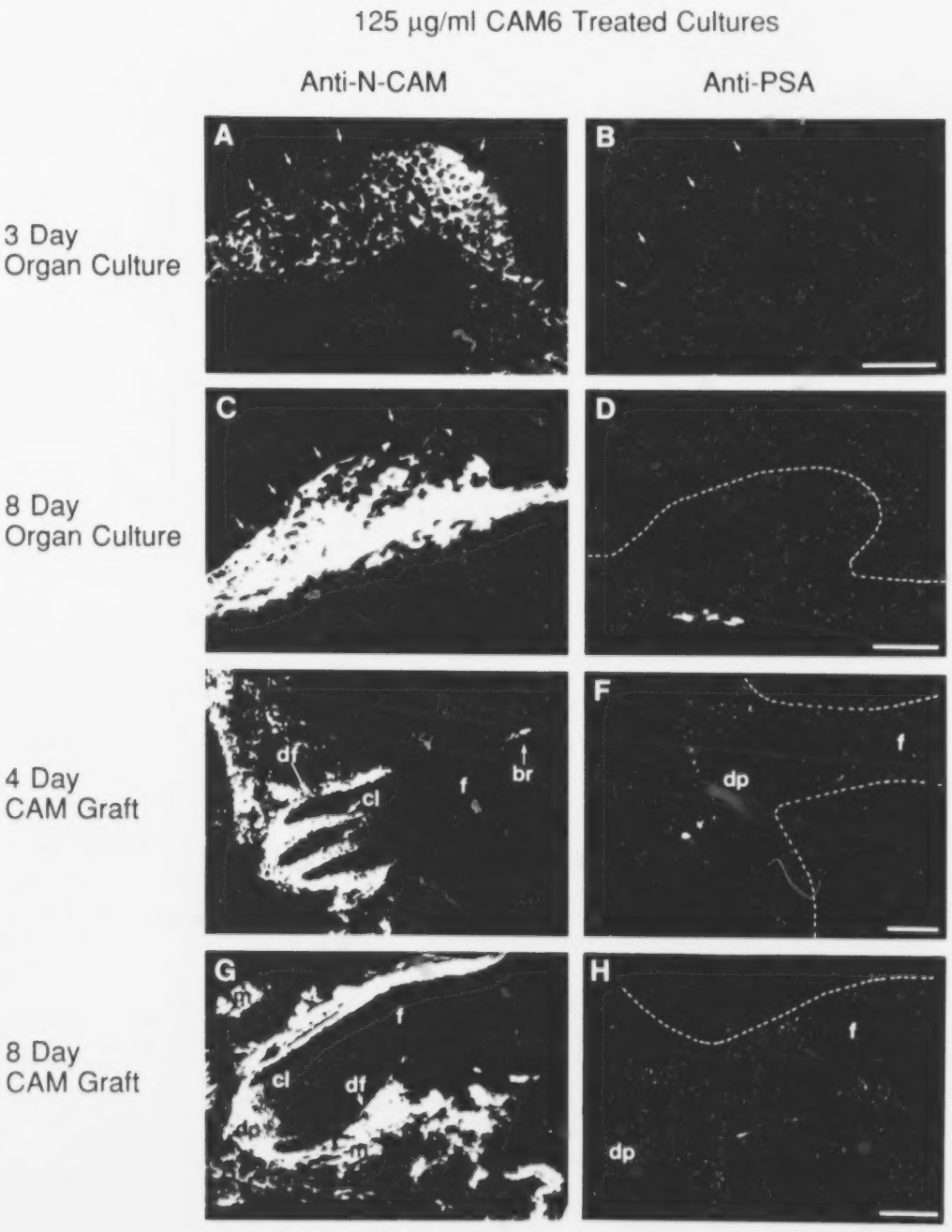
50 U/mL Endo-N Treated Cultures



staining pattern was seen for PSA in wholemounts of CAM6 mAb treated grafts, but was absent in endo-N treated grafts (not shown). Staining with the anti-α-actin mAb showed that the N-CAM and PSA expressing cells in the dermis were smooth muscle (not shown).

The expression of N-CAM and PSA in the endo-N and CAM6 mAb treated 8-day grafts (Figs. III-9 and III-10) was also not affected by the perturbations, except that PSA was completely removed by the endo-N treatment. N-CAM was still expressed on cells of the dermal papilla (dp) and dermal cells of the follicle (dp), and was also present on muscle fibers (m) at the base of the follicles (Figs. III-9G and III-10G). N-CAM was no longer expressed on cells within the filament (f), and was only weakly expressed on cells of the collar (cl), as described in the untreated grafts. In the CAM6 mAb treated grafts, PSA was expressed on cells within the dermis (Fig. III-10H), but this staining was not observed in the endo-N treated grafts (Fig. III-9H). Wholemount staining of N-CAM in both the endo-N and CAM6 mAb treated grafts showed that N-CAM was present on a criss-cross pattern of cells within the dermis, as in the 4-day ChAM grafts (not shown). Wholemount staining showed that PSA was also present on a criss-cross pattern of cells within the dermis of CAM6 mAb treated grafts, but not of endo-N treated grafts (not shown). Staining with the anti-α-actin mAb confirmed that the N-CAM and PSA expressing cells in the dermis were smooth muscle (not shown).

Figure III-10. Immunofluorescent staining of sections of 125 µg/ml CAM6 mAb treated 3-day and 8-day organ cultures and 4-day and 8-day CAM grafts with anti-N-CAM 802 polyclonal antibody (A, C, E, and G) and anti-PSA 5A5 mAb (B, D, F, and H). In all cases, the arrows and dotted lines indicate the outer margin of the epidermis. In the 3-day organ cultures, both N-CAM and PSA are present in the dermal condensations (A-B; single rudiment is shown). In the 8-day organ cultures, N-CAM is expressed more strongly in the basal region of the dermis within the filament-like structure, and is also present on cells of the underlying dermis (C). PSA is restricted to cells in the dermis (D). In the 4-day CAM grafts, N-CAM is present on cells of the dermal papilla (dp), epidermal collar (cl), dermal cells of the follicle (df), and cells of the barb ridges (br) in the filament (f) (E). PSA is restricted to cells in the dermis around the base of the feather follicle (F). The position of the dermal papilla (dp) and filament (f) are shown for orientation. In the 8-day CAM grafts, N-CAM is present on cells of the dermal papilla (dp), dermal cells of the follicle (df), and on muscle fibers in the dermis (m), but is expressed only faintly on cells of the epidermal collar (cl) and is no longer present in the filament (f) (G). PSA is restricted to cells in the dermis around the base of the feather follicle (H). The position of the dermal papilla (dp) and filament (f) are shown for orientation. Scale bars are 50 µm (A-D) and 100 µm (E-H).


125 µg/ml CAM6 Treated Cultures
Anti-N-CAM
Anti-PSA
3 Day
Organ Culture
8 Day
Organ Culture
4 Day
CAM Graft
8 Day
CAM Graft
A
B
C
D
F
G
H
df
cl
f
br
dp
m

Discussion

N-CAM-mediated adhesion is thought to be involved in the formation of many embryonic tissues, including brain, heart, kidney, muscle, and feather, but little is known about the roles of the different N-CAM proteins in the morphogenesis of these structures. To gain insight into the function of individual N-CAM proteins in development, it is useful to determine which N-CAM molecules are restricted to particular tissues or stages of development so that correlations between protein expression and morphogenetic events can be established. Once such correlations are made, theories about the function of individual N-CAM proteins can be developed and subsequently tested by perturbing the adhesion mediated by the particular N-CAM proteins expressed during a morphogenetic event. To this end, we have chosen developing feathers in embryonic chicken as a system in which to examine (a) the N-CAM proteins expressed during formation and patterning of cell populations within an embryonic tissue and, (b) what alterations occur upon perturbing N-CAM-mediated adhesion during this morphogenetic process. We have previously characterized the forms of N-CAM expressed in developing feathers (Marsh and Gallin, 1992). Our results suggested that two 145 kD N-CAM proteins might be involved in epidermal-dermal interaction and in the formation and patterning of the feather rudiments and filaments, and that PSA might regulate cell division in feather buds and be involved in filament formation.

In the present study we set out to determine whether our ideas about the function of N-CAM and PSA in developing feathers were valid by determining what changes in the development of feather

rudiments and filaments occurred upon perturbing N-CAM homophilic binding and removing PSA. Specifically, we cultured explants of stage 30 (E7) skin in the presence of (a) antibodies to inhibit N-CAM homophilic binding, (b) endo-N to remove PSA and, (c) both reagents together, and then examined the short-term and long-term effects of the treatments on rudiment and filament development. We did not observe any changes in the development of rudiments or filaments in either of the single or combined perturbations. This was in contrast to the results of Jiang and Chuong (1992), who treated explants of skin with an anti-N-CAM antibody and found that feather rudiment formation was altered.

One reason why the CAM6 and endo-N treatments may not have caused alterations in feather development was technical problems with the culture system or with the concentrations of antibody and endo-N used. Such problems were largely ruled out by verifying that (a) normal feather structures developed using our culture and grafting systems, (b) N-CAM and PSA were expressed the same way in vitro as in vivo, (c) 50 U/ml of endo-N was sufficient to remove all PSA from the cultures and grafts, (d) the absence of PSA in the endo-N treated cultures and grafts was due to digestion by endo-N and not to unknown factors, (e) the anti-N-CAM antibody CAM6 was able to inhibit N-CAM-mediated adhesion, and (f) CAM6 mAb was able to penetrate and disperse within the skin explants. One potential problem that could not be directly addressed however, was whether the concentration of CAM6 mAb used for perturbation was sufficient to inhibit N-CAM homophilic binding in skin. Based on the aggregation assay with the brain vesicles, 125 μg/ml of CAM6 mAb was

sufficient to inhibit N-CAM-mediated aggregation by 80%. Relative to other N-CAM-containing tissues in the chicken embryo, brain contains the largest amount of N-CAM (Thiery et al., 1982). Thus, the fact that aggregation of the brain vesicles was largely inhibited by 125 µg/ml CAM6 mAb suggested that this concentration of antibody should be sufficient to retard N-CAM homophilic binding in skin, which contains much less N-CAM. In reality, because only a small volume of fluid is present within the skin and the dispersal of the mAb within the tissue is most likely slow, a much lower ratio of antibody to N-CAM may have been present in the skin than in the aggregation assay. For this reason, a higher concentration of mAb may have been required to perturb N-CAM-mediated adhesion in skin than in the aggregation assay. The fact, however, that Jiang and Chuong (1992) observed some change in rudiment development at 125 µg/ml of antibody suggests that this may not be the case. As well, during our preliminary testing, no alterations in development were seen at concentrations of CAM6 mAb as high as 250 µg/ml. Clearly, there is no definitive answer as to whether sufficient CAM6 mAb was used in the current study. To resolve this issue, higher concentrations (250 µg/ml - 1 mg/ml) of CAM6 mAb need to be tested for their ability to alter feather development.

An additional reason as to why our treatments with CAM6 mAb and endo-N might not have caused alterations in feather development was the potential existence of redundant molecular pathways in skin that could have compensated for changes in N-CAM-mediated adhesion. Studies on the function of calcium-dependent cell adhesion molecules (cadherins) in different embryonic tissues

have shown that, even though a cadherin may be expressed in several different tissues, antibodies that perturb the adhesion mediated by this cadherin may cause developmental alterations in one or several of these tissues yet cause no apparent changes in other tissues (Vestweber et al., 1985; Gallin et al., 1986; Hirai et al., 1989a, b). These observations suggest that not all morphogenetic events are mediated by single adhesive mechanisms, and that some processes may make use of redundant adhesive pathways, all of which must be disrupted to discern a developmental role for any of them. An example of this situation occurs in developing mouse whisker, where at least two cadherins are expressed, and where antibodies to both cadherins are required in order to obtain maximal alteration in development (Hirai et al., 1989a, b). Thus, it is quite possible that redundant adhesive pathways may have been present in embryonic chicken skin and may have compensated for changes in N-CAM-mediated adhesion. In opposition to this idea, however, is the perturbation study of Jiang and Chuong (1992), where an antibody against N-CAM alone was shown to alter feather development. Their results suggest that redundant adhesive pathways may not be present in chicken skin, and suggest that perturbing N-CAM alone should be sufficient to alter development. If this is indeed the case, this raises the question as to why our anti-N-CAM antibody (CAM6) did not cause similar alterations in feather development. This question will be dealt with later in this discussion.

A third reason why our CAM6 mAb and endo-N treatments might not have caused alterations in feather development is the fact that the presence of a molecule at a specific time and place does not

necessarily imply that the molecule has a function at that time and place. Thus, even though N-CAM and PSA were present in the skin cultures and grafts, it was possible that these molecules were not involved in feather development at the stages we examined. This possibility seems unlikely since a time span of 8 days, covering both rudiment and filament formation, was examined. As well, Jiang and Chuong (1992) showed that their anti-N-CAM antibody altered feather development at the stage of rudiment formation, a time period covered by our perturbation study. Unlike in our study, however, Jiang and Chuong (1992) used stage 33 (E8) skin explants for their antibody perturbations, whereas we used stage 30 (E7) explants instead. At stage 33 (E8), several rows of feather rudiments are already established in the skin. We have not yet determined whether this variation in time of treatment was significant in terms of the different outcomes of the two perturbation studies.

Overall, the differences in effect of the two anti-N-CAM antibodies, and the lack of effect of removing PSA, can not be adequately explained by technical problems with our approach, the existence of redundant adhesive pathways, or lack of function of N-CAM and PSA at the stages that were perturbed.

Studies on the structure of the N-CAM molecule (Cunningham et al., 1983; Cole et al., 1986; Frelinger and Rutishauser, 1986; Rutishauser and Goridis, 1986; Watanabe et al., 1986) have shown that each N-CAM protein can be divided into three regions: (a) an amino-terminal domain that contains both a homophilic binding site and a heparin binding site, (b) a central domain that contains the polysialic acid attachment sites and, (c) a membrane-associated

domain extending to the carboxyl terminus. Studies have also shown that N-CAM interacts not only with additional N-CAM on other cells, but also with extracellular matrix (ECM) molecules (Cole and Glaser, 1986; Cole et al., 1986; Probstmeier et al., 1989, 1992; Acheson et al., 1991; Aoki et al., 1991) and with other molecules on the cell surface (Kadmon et al., 1990a, b). The fact that individual regions of an N-CAM molecule contain distinct binding and attachment sites suggests that these regions may have specific functions in mediating homophilic binding, interaction with ECM molecules, and interaction with other proteins (Cunningham et al., 1983; Cole and Glaser, 1986; Frelinger and Rutishauser, 1986; Watanabe et al., 1986; Probstmeier et al., 1989, 1992; Kadmon et al., 1990a). If distinct functional regions are present in an N-CAM molecule, the possibility exists that certain regions of the molecule may play a more important role in development at a particular stage or in a particular tissue. As well, the possibility exists that different regions of the same N-CAM molecule may be able to compensate for loss of function in another part of the molecule. The idea that distinct functional regions are present within an N-CAM molecule is supported by observations that mAbs which recognize different sites on N-CAM have varied effects on homophilic binding (Frelinger and Rutishauser, 1986; Watanabe et al., 1986). In particular, mAbs which recognize the heparin binding site in the amino-terminal domain usually cause inhibition of N-CAM binding, whereas mAbs that recognize the homophilic binding site in this domain can cause either inhibition or enhancement of binding (Watanabe et al., 1986). In addition, combinations of mAbs have a greater effect on N-CAM homophilic binding than single mAbs alone

(Frelinger and Rutishauser, 1986; Watanabe et al., 1986). These findings are consistent with the idea that specific sites on the N-CAM molecule have distinct functions in mediating homophilic binding, and suggest that several sites on the molecule may need to be bound by mAbs in order to achieve maximal loss of function. Thus, there is nothing to rule out the idea that different sites on the N-CAM molecule may have more important functions at particular stages of development or in particular tissues.

Keeping in mind that an N-CAM molecule may have distinct functional regions and that one or several of these regions may be more important at a given developmental stage, there are several possible reasons why our antibody did not alter feather development yet the antibodies used in the Jiang and Chuong (1992) perturbation experiment did. In particular, even though CAM6 mAb inhibited N-CAM homophilic binding in the aggregation assay, the possibilities existed that (a) the N-CAM molecules in skin were able to compensate for the loss of binding and were able to re-establish normal homophilic binding by using sites on the proteins other than the site bound by CAM6 and, (b) the binding function of N-CAM was either not important or less important than other functions of N-CAM in skin such as interaction with ECM or with other cell surface proteins. Thus, if our antibody bound to a region on the skin N-CAM proteins that did not mediate an important function during feather morphogenesis, it would not cause alterations in development. In contrast, if the antibodies used in the Jiang and Chuong (1992) study bound to different regions of the skin N-CAM proteins that did mediate an important function, then alterations in development would be

caused. In support of this idea, the antibodies used in the Jiang and Chuong investigation were polyclonal. Thus, their antibodies most likely bound to multiple sites on the skin N-CAM proteins and may therefore have perturbed more than just homophilic binding. To determine if this was the case, it would be necessary to repeat the perturbation studies carried out in this thesis using the same antibodies as in the Jiang and Chuong study. As well, since mAbs that bind to different regions of N-CAM are available (Watanabe et al., 1986), it would be useful to determine if different effects on feather development are caused by treating skin explants with these mAbs, both singly and in combinations.

While the existence of distinct functional regions on the skin N-CAM proteins may explain why CAM6 mAb did not alter feather development, it does not afford as good an explanation as to why removal of PSA did not alter feather development. Unlike the anti-N-CAM antibody treatment, where a single site on the skin N-CAM proteins was bound and perturbed, our treatment with endo-N was an all-or-none event; we showed that 50 U/ml endo-N completely removed all detectable PSA from N-CAM in the dermal condensations. Put simply, since no PSA was left on the skin N-CAM, whatever developmental function the PSA had could no longer exist. PSA is a large, negatively-charged carbohydrate that inhibits contact between the plasma membranes of apposed cells (Rutishauser et al., 1988). At present, no known domain of the N-CAM proteins has a similar function. Thus, the fact that endo-N treatment did not alter feather development suggested that PSA was not directly involved in feather morphogenesis, at least not at the stages that we perturbed.

An alternative function for the PSA detected in the dermal condensations in vivo and in vitro deals with the development of smooth muscle cells of the feather erector muscles. We have shown that, in vivo, PSA is present on N-CAM in the dermal condensations for only a short period of time (stages 35-36, E9-E10), and is then expressed on developing smooth muscle within the dermis from stage 37 (E11) until the latest stage that we examined (stage 44, E18) (Marsh and Gallin, 1992). This observation suggested that the PSA in the condensations might be present on smooth muscle precursor cells, which subsequently migrated into the dermis and formed into feather erector muscle. This idea was not easily tested in vivo however, because a clear correlation between the PSA-expressing cells present in the condensations and subsequently present in the dermis could not be established; the PSA-expressing cells in the condensations did not stain with either the anti-α-actin mAb specific for smooth muscle or with an anti-tropomyosin mAb that recognized skeletal muscle (Marsh and Gallin, 1992). Hence their development into the feather erector muscles could not be followed and confirmed. Surprisingly, this problem was overcome by using organ cultures of skin because erector muscle continued to develop in vitro (based on staining with anti-α-actin mAb), indicating that the erector muscle cells had to be present within the dermis at the time the skin was removed from the embryo (stage 30, E7). Moreover, an apparent correlation between the PSA-expressing cells in the condensation and in the dermis could be established in vitro because the smooth muscle cells of the erector muscle developed slower in the 8-day

organ cultures than in vivo. This correlation can be seen by comparing the expression of PSA in Figs. III-2B and III-2E, and in Figs. III-8B and III-8E. The PSA-expressing cells in the condensations of the 3-day organ cultures (Figs. III-2B and III-8B) did not stain with anti-α-actin mAb (not shown), but these cells in the 8-day cultures did stain with anti-α-actin mAb (not shown; identical to Figs. III-2E and III-8E). This suggested that the PSA in the 3-day condensations was present on smooth muscle precursor cells, and that these cells eventually developed into the mature smooth muscle cells of the feather erector muscles, which we showed expressed PSA in vivo (Marsh and Gallin, 1992). If this was indeed the case, then the removal of PSA from the skin explants with the endo-N treatment would not be expected to cause any changes in feather development because the PSA would have had no direct function in that process. However, removal of PSA might be expected to alter the smooth muscle development in the dermis. Unfortunately, when comparing the formation and organization of smooth muscle in the same stage untreated, endo-N treated, and CAM6 mAb treated cultures and grafts by staining with anti-N-CAM 802 polyclonal antibody, no apparent changes in these parameters were observed (not shown). The effects of perturbing N-CAM-mediated adhesion in the dermal smooth muscle needs to be examined in greater detail in future investigations.

Although removing PSA from the skin explants using endo-N did not lead to any alterations in feather formation, an apparent decrease in the amount of N-CAM expressed in the endo-N treated cultures and grafts was detected using Western blot analysis

(Fig. III-4D, lanes 2 and 5 compared to lanes 1, 3, and 4). We showed that the removal of PSA from the endo-N treated cultures and grafts was due to digestion by the endo-N and not to toxicity. For this reason, the decreased amount of N-CAM detected in the endo-N treated cultures and grafts could also not be due to toxic side-effects of the endo-N. With the LDL receptor, there is evidence that increased protein turnover is associated with the removal of carbohydrate from the protein, suggesting that carbohydrate may serve to stabilize membrane molecules and hence increase surface expression (Kozarsky et al., 1988). Thus, it is possible that removal of PSA from N-CAM may decrease the amount of N-CAM expressed at the cell surface. At present, there is no evidence for this effect in the literature. Additional studies need to be done to determine whether or not PSA actually does modulate the amount of N-CAM expressed at the cell surface.

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CHAPTER IV

Discussion and Conclusions

Results from a large number of perturbation experiments suggest that the N-CAM family of proteins plays an important role in development by mediating interactions between cells and by regulating primary cellular processes. While early investigations into the structure of N-CAM suggested that the N-CAM family was composed of three different proteins (Hoffman, 1982; Cunningham et al., 1983; Lyles et al., 1984), it is now clear that N-CAM actually consists of a much larger family containing the three major N-CAM proteins plus multiple variants of these proteins (Dickson et al., 1987; Prediger et al., 1988; Small et al., 1988; Santoni et al., 1989; Thompson et al., 1989; Small and Akeson, 1990; Reyes et al., 1991; Zorn and Krieg, 1992). These variant proteins are created by the insertion of short, extra peptide sequences into the extracellular region of the major proteins (see Fig. I-3). The variant proteins appear to have distinct spatiotemporal expression patterns in the embryo (Small et al., 1988; Small and Akeson, 1990; Reyes et al., 1991), suggesting that each variant may have a specific function and that regulation of N-CAM-mediated adhesion may occur at a very fine level during embryogenesis. At present, however, differences in function between the large number of N-CAM proteins have not been demonstrated.

To further our understanding about the role of N-CAM-mediated adhesion in development, it will be necessary to determine the functions of the individual N-CAM proteins. As a first step towards

achieving this goal, it is useful to determine the types of N-CAM expressed in individual embryonic tissues at specific stages of development and to correlate the expression of particular N-CAM proteins with the occurrence of different morphogenetic events. The establishment of such correlations will provide ideas about the function of particular N-CAM proteins during morphogenesis, and also serve as a framework on which to build experiments to look at the function of specific N-CAM proteins during developmental processes. To this end, we have used developing feathers in embryonic chicken skin as a system in which to (a) characterize the N-CAM proteins expressed during the formation and patterning of cell populations within a tissue and, (b) carry out a preliminary investigation into the effects of perturbing N-CAM-mediated adhesion during this developmental process.

Upon initial examination, N-CAM in skin appeared as a single 145 kD protein, encoded by a single 6.4 kb mRNA, compared to the 140 kD N-CAM protein and 6.1 kb mRNA in brain. The larger size of the skin protein and mRNA compared to those in brain suggested that alternative exons might be present in skin N-CAM and account for the larger size of the protein and mRNA. Polymerase chain reaction (PCR) was therefore used to determine whether known (see Fig. I-3) or unknown alternative exons were spliced into the skin N-CAM mRNA. PCR showed that two N-CAM mRNAs, differing by the presence versus absence of a 93-bp insert between exons 12-13, were present in skin. This meant that the single 6.4 kb mRNA band detected on Northern blots actually consisted of two 6.4 kb mRNAs that could not be resolved using our gel system. As well, this meant

that the single 145 kD protein band detected on Western blots probably consisted of two 145 kD proteins that also could not be resolved. Because only a single mRNA band was detected on the Northern blots, the 93-bp insert could not account for the larger size of the 6.4 kb skin mRNAs compared to the 6.1 kb mRNA in brain. Likewise, because only a single protein band was detected on the Western blots, the 93-bp insert could also not account for the larger size of the 145 kD skin proteins compared to the 140 kD protein in brain.

Alternative explanations as to the larger size of the two skin 6.4 kb mRNAs compared to the 6.1 kb mRNA in brain were the use of a different polyadenylation site at the 3' end of the skin mRNAs (Goridis and Wille, 1988) or the use of a different transcriptional start site at the 5' end of the skin mRNAs than in the brain mRNAs (Barthels et al., 1987; Hirsch et al., 1990; Colewell et al., 1991; Brackenbury, 1992). To determine if different polyadenylation sites were being used in the skin versus the brain mRNAs, RACE PCR (Frohman et al., 1988) was used to compare the length of the 3' untranslated regions of the skin and brain transcripts. No difference in the length of this region in the skin and brain mRNAs was found, indicating that a different polyadenylation site could not account for the larger size of the skin transcripts. To determine if a different transcriptional start site was used in the skin versus the brain mRNAs, primer extension was used to compare the length of the 5' region of the transcripts in both tissues. Unfortunately, results obtained with primer extension were not conclusive due to the fact that this technique is confounded by the high GC content (85%) of

the 5' end of N-CAM. In spite of these inconclusive results, use of an alternative transcriptional start site in the skin mRNA remains a likely explanation for the larger size of the 6.4 kb skin mRNAs compared to the 6.1 kb brain mRNA. At least two different start sites have been found in mouse brain N-CAM and appear to be used indiscriminately by different cell types during development (Barthels et al., 1987; Hirsch et al., 1990). Recent evidence suggests that different transcriptional start sites may also be present in chicken N-CAM (Colewell et al., 1991; Brackenbury, 1992). Interestingly, about 330-bp upstream of the major start site known to be used in chick brain N-CAM is a TATA-box that represents another potential start site (Brackenbury, 1992). The position of this TATA-box corresponds well with the observed 300-bp size difference between the skin and brain N-CAM mRNAs. It still remains to be shown, however, that this TATA-box is used in chicken N-CAM and that use of this site accounts for the larger size of the skin N-CAM mRNAs.

An alternative explanation as to the larger size of the 145 kD skin N-CAM proteins compared to the 140 kD protein in brain was the presence of N-linked or O-linked carbohydrates on the skin proteins (Crossin et al., 1984; Cunningham et al., 1987; Walsh et al., 1989). To determine if this was the case, lectin blot analysis was carried out using lectins that recognized N-linked (wheat germ agglutinin) or O-linked carbohydrate (peanut lectin, jackfruit lectin) (Allen et al., 1973; Naquta and Berger, 1974; Lotan et al., 1975; Mahanta et al., 1990). Analysis of the carbohydrate attached to the skin proteins by using these lectins showed that N-linked carbohydrate was present

on the skin N-CAM proteins but that O-linked carbohydrate was not present. To verify that N-linked carbohydrate was indeed the basis of the larger size of the skin N-CAM proteins compared to the 140 kD protein in brain, N-linked carbohydrate was removed from skin and brain N-CAM by digestion with endoglycosidase-F (endo-F) (Elder and Alexander, 1982) and the relative decrease in molecular weight of the skin and brain proteins was compared. Upon digestion with endo-F, the 140 kD brain protein decreased in molecular weight by about 8 kD. In contrast, the 145 kD skin proteins decreased in molecular weight by about 13 kD, and were a similar size to the endo-F digested 140 kD brain protein. This indicated that N-linked carbohydrate did indeed account for the size difference between the 145 kD N-CAM proteins in skin and the 140 kD protein in brain.

Because several sites for N-linked attachment are known to be present on brain N-CAM but only three of these sites appear to be used (Crossin et al., 1984; Cunningham et al., 1987), the question remained as to whether the larger size of the skin proteins was due to (a) an increased number of N-linked carbohydrate chains on these proteins compared to the brain proteins, i.e. extra sites being N-glycosylated on the skin N-CAM proteins, or (b) the same number of sites were used, but more carbohydrate was present per chain. To differentiate between these two possibilities, skin N-CAM was digested with serial dilutions of endo-N to sequentially remove N-linked chains from the proteins. Using this technique, three N-linked chains were found to be present on the skin N-CAM proteins, the same number as described for brain N-CAM. This indicated that the larger size of the skin N-CAM proteins was due

to the presence of more N-linked carbohydrate per chain and not to glycosylation of additional sites on the proteins.

Having determined that two 145 kD N-CAM proteins were present in skin and having investigated the structural features of these proteins, the next step was to examine in detail the expression pattern of N-CAM in developing feathers and also the expression pattern of polysialic acid (PSA), a carbohydrate known to modulate N-CAM-mediated adhesion (Hoffman and Edelman, 1983; Rutishauser et al., 1988; Acheson et al., 1991). Using staining of skin whole-mounts and sections, N-CAM was found to be expressed on cells involved in the formation of the feather rudiments and feather filaments, in particular on cells of the dermal condensations, cells of the epidermal collar, and on cells of the marginal and axial plates in the filaments, as described previously (Chuong and Edelman, 1985a). In addition, N-CAM was also found to be expressed on cells of the epidermis early on in feather development, and to become localized to cells of the epidermal placodes (Marsh and Gallin, 1992). We showed that PSA was expressed on only a subset of the cells that expressed N-CAM, in particular on cells of the dermal condensations from stage 35-36 (E9-E10) and on smooth muscle cells in the dermis from stage 37 (E11) until the latest stage we examined (stage 44, E18). The expression of PSA in the dermal condensations appeared to be localized to the cells that undergo the least cell division at the onset of filament formation (Wessells, 1965; Desbien et al., 1991).

To briefly summarize the potential significance of the findings described above, characterization of N-CAM in developing feathers shows that two N-CAM proteins, differing by the presence versus

absence of a 31 amino acid insert, are present in developing feathers, and suggests that the function of these proteins might be modulated by (a) attachment of N-linked carbohydrate (other than PSA) to the proteins and (b) variation in the amount of PSA attached to the proteins. Our findings also suggest that a different transcriptional start site may be used in skin N-CAM. In Chapter II of this thesis, it was mentioned that a similar-sized 145 kD protein and 6.4 kb mRNA were found in skin, skeletal muscle, and heart N-CAM. While not proven, it is possible that all of these non-neuronal tissues may use the different transcriptional start site in their mRNA, and that this site may be involved in tissue-specific regulation of the 145 kD N-CAM proteins in non-neuronal tissues. Examination of the expression pattern of N-CAM and PSA in developing feathers shows that N-CAM is expressed on cells that are undergoing inductive interaction, on cells that are dividing, and may be expressed on cells that undergo death, and that PSA is expressed only transiently on N-CAM in the dermal condensations (stage 35-36, E9-E10) and is then expressed on N-CAM on smooth muscle cells in the dermis from stage 37 (E11) until at least stage 44 (E18). The expression of PSA in the feather rudiments coincides with the region of the rudiments that undergoes the least cell division as elongation of the feather filaments begins.

Knowing the forms of N-CAM and the expression pattern of N-CAM in developing feathers, the next step was to formulate ideas about the function of the N-CAM proteins in feather morphogenesis. Because N-CAM in developing feathers is expressed on cells that

are undergoing inductive interactions, cells that are dividing, and perhaps on cells that die, it is possible that the N-CAM proteins may be involved in these processes and may play a role in directing feather formation. If this is the case, then perturbing N-CAM-mediated adhesion in feathers may cause changes in the formation and patterning of the feather rudiments and changes in the development of the filaments, perhaps by altering the primary cellular processes. In support of this idea, a recent study that perturbed N-CAM-mediated adhesion in developing feathers using an anti-N-CAM antibody (Jiang and Chuong, 1992) showed that normal formation and patterning of the feather rudiments was altered. This study, however, did not look at the underlying basis for the altered rudiment development, i.e. changes in the primary cellular processes, nor at the effect of removing PSA. For this reason, we carried out a more detailed investigation into the effects of perturbing N-CAM-mediated adhesion in developing feathers.

The objective of the perturbation study described in this thesis was to look in detail at the effects on feather development of inhibiting the homophilic binding function of the skin N-CAM proteins and of removing PSA. To this end, skin explants were treated with a monoclonal antibody (mAb) known to inhibit N-CAM homophilic binding and also with endoneuraminidase-N (endo-N) to remove PSA, and the short-term and long-term effects on rudiment and filament formation were examined. It was expected that abnormal rudiment development would be caused by the anti-N-CAM antibody, as described in the Jiang and Chuong (1992) study, and that removal of PSA might alter normal cell division in the

rudiments and lead to abnormal filaments. Surprisingly, neither the antibody nor the endo-N treatment led to any apparent changes in the morphology or histology of the feather rudiments or filaments, suggesting that the normal cellular processes which occur during feather morphogenesis were not altered.

As discussed in Chapter III of this thesis, there were a number of possible reasons why the antibody and endo-N treatments did not alter feather development, including technical problems, the existence of redundant adhesive pathways in skin, and the fact that the time of expression of N-CAM does not necessarily coincide with the time that N-CAM is involved in feather development. Technical problems were largely ruled out by verifying that (a) normal feather structures developed using our culture and grafting systems, (b) N-CAM and PSA were expressed the same way in vitro as in vivo, (c) 50 U/ml of endo-N was sufficient to remove all PSA from the cultures and grafts, (d) the absence of PSA in the endo-N treated cultures and grafts was due to digestion by endo-N and not to unknown factors, (e) the anti-N-CAM mAb inhibited N-CAM homophilic adhesion, and (f) the anti-N-CAM mAb was able to penetrate and disperse within the skin explants. The existence of redundant adhesion pathways that might have compensated for the loss of N-CAM homophilic binding in skin also seemed unlikely, since antibodies against N-CAM were found to alter feather morphogenesis in a previous study (Jiang and Chuong, 1992) and showed that normal N-CAM-mediated adhesion was crucial for proper feather development. Likewise, the possibility that N-CAM expression and function did not coincide temporally was also

unlikely because the time span examined in the current perturbation study was from stage 30 (E7) to stage 42 (E16), a period covering both rudiment and filament formation. One difference, however, between the current study and the Jiang and Chuong (1992) study was the time of initial application of antibody to the skin explants; in the current study the skins were exposed to antibody at stage 30 (E7) whereas in the other study the skins were exposed to antibody at stage 33 (E8). At stage 30, feather rudiments are just starting to appear in the dorsal skin. In contrast, at stage 33, multiple rows of feather rudiments are already established. The possibility therefore exists that this difference in the time of antibody application might have led to the different results of the two studies. This possibility remains to be tested.

An additional reason why the antibody treatment in the current study may not have caused alterations in feather morphogenesis was the possibility that different regions of the skin N-CAM proteins mediated specific functions such as homophilic binding, interaction with extracellular matrix (ECM), or interaction with other cell surface proteins. Structural studies of N-CAM (Cunningham et al., 1983; Cole et al., 1986; Frelinger and Rutishauser, 1986; Watanabe et al., 1986) have shown that the N-CAM molecule consists of several distinct regions that contain the known cell-cell binding site, cell-ECM binding site, and carbohydrate attachment sites. The possibility therefore existed that these different binding and attachment sites might have had varied importance at different stages of feather development. Additionally, the possibility existed that different regions of the skin N-CAM proteins might have compensated for loss

of function in another part of the proteins. Keeping these ideas in mind, even though the antibody used in the current perturbation experiment was shown to inhibit N-CAM homophilic binding, this function of N-CAM may not have been important for normal feather development, or other regions of the skin N-CAM proteins may have compensated for the loss of binding. If either, or both, of these possibilities were true, then alterations in feather development would not have been caused by our antibody. In contrast, if the antibodies used in the Jiang and Chuong (1992) study bound to different regions of the skin N-CAM proteins that did mediate important functions, or if the function of the bound regions could not be compensated for, then alterations in development would have been caused. In support of this idea, the antibodies used in the Jiang and Chuong study were polyclonal, and therefore likely bound to many different regions on the skin N-CAM proteins. Consequently, it is entirely possible that their antibodies altered different functions of the skin N-CAM proteins than our antibody and therefore had a different effect on feather morphogenesis.

In terms of the endo-N treatment, one explanation as to why removal of PSA did not lead to any changes in feather morphogenesis was the possibility that the PSA present in the dermal condensations was not involved in feather morphogenesis but was instead involved in development of the feather erector muscles within the dermis. Staining of the organ cultures and ChAM grafts with an α-actin mAb showed that smooth muscle cells continued to develop within the dermis in vitro. This indicated that the cells involved in erector muscle formation were present in the skin at the time of removal

from the embryo (stage 30, E7) and subsequently organized themselves into the criss-cross pattern characteristic of mature erector muscle. In the 3-day organ cultures, PSA was expressed in a crescent-shaped pattern in the cephalic region of the feather rudiments. In the 8-day organ cultures, PSA was still expressed in the same crescent-shaped pattern, but bands of cells interconnecting the crescent-shaped areas in different feather rudiments also expressed PSA. This observation suggested that the PSA-staining cells in the feather rudiments of the 3-day cultures might represent smooth muscle precursor cells, and that these cells might not be directly involved in the formation of feathers. If this was indeed the case, then the removal of PSA from the skin explants would not have altered feather development. However, the removal of PSA might have altered the normal development of the smooth muscle cells themselves. Unfortunately, no apparent changes in the development or organization of the smooth muscle cells were observed in either the endo-N or CAM6 mAb treated cultures and grafts.

Clearly, a large number of questions remain about the function of N-CAM in feather morphogenesis. To further our understanding about the role of N-CAM in this developmental process, future experiments should determine whether (a) the two N-CAM proteins in skin have distinct spatiotemporal expression patterns during feather morphogenesis, (b) the 93-bp insert modulates the function of N-CAM in skin, (c) the N-linked carbohydrate present on the skin proteins modulates their function and, (d) antibodies that recognize different regions of the N-CAM proteins have different effects on feather morphogenesis when used both singly and in combination.

Although not directly related to feather development, it would also be interesting to examine in greater detail the function of N-CAM-mediated adhesion in the formation of the feather erector muscles.

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